THE FECAL PORPHYRIN PROFILE IN RIVER OTTERS AS A BIOMARKER OF THE EFFECTS OF OIL EXPOSURE

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ABSTRACT

The use of profiles of fecal porphyrins in river otters as a biomarker of the effects of oil exposure was investigated. A high-performance liquid chromatography method was developed to quantify porphyrins and compared to a spectrofluorometric method. Profiles of porphyrins were characterized in fecal samples collected at oiled and nonoiled areas in Prince William Sound following the *Exxon Valdez* oil spill. Levels of porphyrins in fecal samples indicated a temporal pattern of physiological stress and recovery similar to that described by other biomarker studies. Profiles of porphyrins were also characterized in fecal samples from southeast Alaska and contributed to the establishment of a baseline for fecal porphyrin excretion in coastal river otters. No significant difference in porphyrin excretion occurred between treatment groups of river otters experimentally dosed with oil. The experimental results suggest that heme metabolism in river otters may be effected by captive conditions.

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INTRODUCTION

The *Exxon Valdez* oil spill of March, 1989 severely impacted near-shore habitats within the marine ecosystem of Prince William Sound, Alaska, USA. Several research programs were developed by the Exxon Valdez Oil Spill Trustee Council that focused on the dynamics of the marine ecosystem in Prince William Sound to evaluate recovery of species and resources effected by the oil spill. The Near-Shore Vertebrate Predator project (NVP) is one such program that was established in 1995 to study the recovery of the coastal habitat by focusing on four different predator species: river otters, pigeon guillemots, sea otters, and harlequin ducks. To evaluate the recovery status of these four species, NVP research has encompassed specific health factors as well as nutrition and population dynamics in an attempt to determine if direct exposure to oil or oil related loss of food and habitat is effecting recovery (Holland-Bartels et al., 1998).

River otters (*Lutra canadensis*), serve as an excellent sentinel species for the evaluation of the chronic effects of exposure to persistent levels of oil within the coastal ecosystem of Prince William Sound because these mustelids occupy a high trophic position within the marine ecosystem and feed on intertidal and subtidal organisms (Larsen, 1984; Bowyer et al., 1994; Ben-David et al., 1998). River otters are ubiquitous within Prince William Sound as well as the Gulf of Alaska (Bowyer et al., 1995), and therefore sampling protocols at both oiled and nonoiled sites may be readily developed. Also, river otters have been shown to serve as effective indicators of the presence of a number of environmental contaminants (Wren et al., 1980; Wren, 1985; Duffy et al., 1993; Mason, 1993; Mason et al., 1993; Ropek and Neely, 1993; Bowyer et al., 1994; Duffy et al., 1994a, b; Francis and Bennet, 1994; Bowyer, 1995; Blajeski et al., 1996; Duffy et al., 1999).

As a sentinel species in Prince William Sound, river otters serve not only as marker of the presence and bioavailability of persistent levels of oil in the coastal ecosystem, but they also serve as indicators of the potential effects oil will have on the

physiology and health of coastal organisms. River otters feed on both fish and shellfish, both of which were shown to accumulate oil following the oil spill. River otters, therefore, may be exposed to oil through their diet (Babcock et al., 1990; Meachum and Sullivan, 1990; Babcock and Short, 1996; Collier et al., 1996). River otters can come in direct contact with oil remaining in sediments (Babcock et al., 1990; Kvenvolden et al., 1993; Babcock and Short, 1996) and subsurface sea water receiving oil from shoreline and sediment reservoirs of oil (Short and Harris, 1995). River otters may ingest oil that has accumulated on their pelage while grooming (Baker et al., 1981; Duffy et al., 1999).

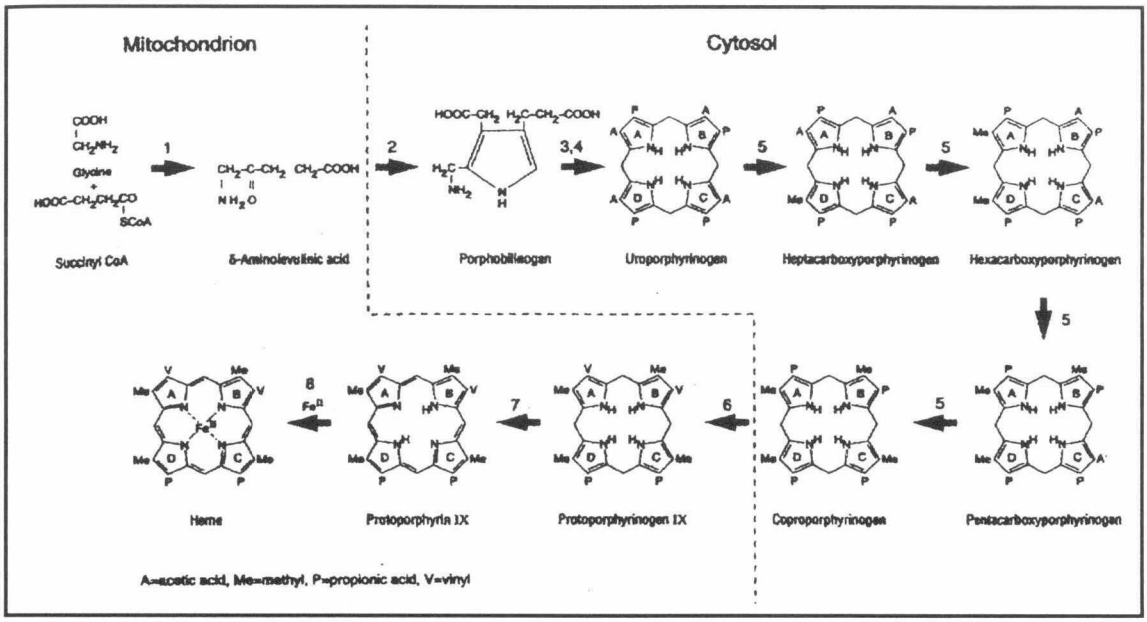
The measurement of contaminant availability is not sufficient, however, to evaluate toxic effects on an organism, particularly for compounds such as polyaromatic hydrocarbons (PAH), components of crude oil, which are rapidly metabolized and excreted by vertebrate organisms (Cappuzo, 1987; Leadly et al., 1999). Furthermore, only a small fraction of the compounds within the PAH class have been toxicologically evaluated (Fazio and Howard, 1983). Research on the health and recovery status of river otters within oiled areas of Prince William Sound, therefore, includes the measurement of biochemical and physiological indicators, or biomarkers, of the effects of oil exposure.

Biomarkers include components of an organism's compensatory and detoxifying mechanisms such as heat shock protein induction and the cytochromes P-450 mono-oxygenase systems as well as components of the immune system (Rand, 1995). Biomarkers also include products of cellular damage or malfunction such as hemoglobin adducts, DNA adducts, and porphyrins (Hugget et al., 1992). Biomarkers are useful for measuring the health of an organism and may facilitate the integration of the toxicokinetic interactions resulting from exposure to a mixture of chemicals (Fossi et al., 1994). Biomarkers may also provide and early warning signal of long-term and community and population effects (Rand, 1995). This is particularly useful for biomonitoring programs in Prince William Sound that focus on recovery factors of species based upon their interrelationships with aspects of the entire marine ecosystem. The connection between biomarkers and community or population effects may be described by a hierarchy of

biological response (Fossi et al., 1994). Biochemical responses to exposure to foreign chemicals result in cellular and physiological alterations that effect the health and survivorship of individuals. Effects on the fitness of an individual, particularly, the ability to reproduce, may effect the viability of the population.

There has been recent interest in the development of nondestructive biomarkers, which are biochemical indicators of response that are measured without harming the organism (Fossi and Leonzio, 1994). Sources of nondestructive biomarkers include blood, urine, and feces. There are several advantages to the use of nondestructive biomarkers that are relevant to NVP research objectives. Population numbers are not compromised by sacrificing individuals for biological sampling and restrictions against studying higher vertebrates may be avoided (McCarthy, 1994). Also, recapture of unharmed organisms facilitates time sequence studies, which are useful for evaluating chronic effects of oil exposure (Fossi et al., 1994). This thesis research project investigated the use of the profile of fecal porphyrins as a nondestructive biomarker of the effects of oil exposure on heme metabolism in river otters. Although porphyrins may be measured in urine and feathers as well as feces, the use of fecal samples allows "noninvasive" as well as nondestructive biomarker measurements and precludes the need for capturing and handling river otters for biological sampling (Fossi and Leonzio, 1994; Fossi et al., 1996; Wasser et al., 1996; Malakoff, 1997).

Heme biosynthesis proceeds through a series of tetrapyrollic intermediates called porphyrinogens, which are subsequently oxidized to porphyrins when produced in excess (Fig. 1.1). Approximately 85% of heme metabolism in mammals occurs in the erythropoetic tissue where heme is primarily incorporated into hemoglobin; the majority of the remainder of heme metabolism occurs in the hepatic tissue where heme is incorporated into enzymes that facilitate many endogenous functions (Voet and Voet, 1995). The hepatic tissue in mammals is also one of the primary sites of metabolism of foreign chemicals and there is, therefore, a strong proximal link between the metabolism of foreign chemicals and potential interference with heme metabolic function (Duffus and Worth,



Adapted from Woods et al. 1993.

Figure 1.1. The heme biosynthetic pathway. Enzyme steps are: 1, δ-aminolevulinic acid synthase; 2, δ-aminolevulinic acid dehydratase; 3-4, uroporphyrinogen synthase-uroporphyrinogen cosynthase; 5, uroporphyrinogen decarboxylase; 6, coproporphyrinogen oxidase; 7, protoporphyrinogen oxidase; 8, ferrochelatase.

1996). Alterations of heme metabolism may occur as a result of direct inhibition of enzyme function within the metabolic pathway leading to an accumulation of the preceding porphyrinogen precursor or as a result of the occurrence of an oxidative stress mechanism which may directly oxidize porphyrinogens to porphyrins (Woods, 1989). Excretion products in hepatic tissue, such as porphyrins, follow a biliary excretion route for ultimate removal from the organism, and therefore, may be measured in fecal samples (Duffus and Worth, 1996). Elevated levels of porphyrins in fecal samples, therefore, may serve as an indicator of the alteration of hepatic heme metabolism (DeMatteis and Lim, 1994) (Fig. 1.2). Furthermore, characterization of the entire profile of porphyrins in fecal samples may facilitate identification of specific enzyme dysfunction as well as more subtle changes in porphyrin excretion due to contaminant exposure, which may be quantitatively indistinguishable from normal porphyrin excretion levels (Marks, 1985).

Current methods for the analysis of porphyrins in biological samples involve separation with high-performance liquid chromatography and fluorescence detection (Lim, 1991). Porphyrins have characteristic fluorescence spectra, which allows selective and sensitive detection in complex biological samples (DeMatteis and Lim, 1994). Recent trends in method development for the analysis of porphyrins have included reduction in chromatographic separation time and increased sensitivity of fluorescence detection. The development of more rapid and sensitive analytical techniques allows the analysis of large numbers of samples in the context of comprehensive biomonitoring programs designed to assess chronic effects of exposure to low levels of contaminants persistent in the environment.

Chemically induced porphyrinopathies have been well documented in humans as well as laboratory animals (Miranda et al., 1987; Silbergeld and Fowler, 1987; Fossi et al., 1996; Daniell et al., 1997; Franklin et al., 1997). Although many porphyrinopathies in humans are described as toxicogenetic, heme metabolism may be altered in individuals without a genetic predisposition to heme metabolism disfunction upon exposure to a wide

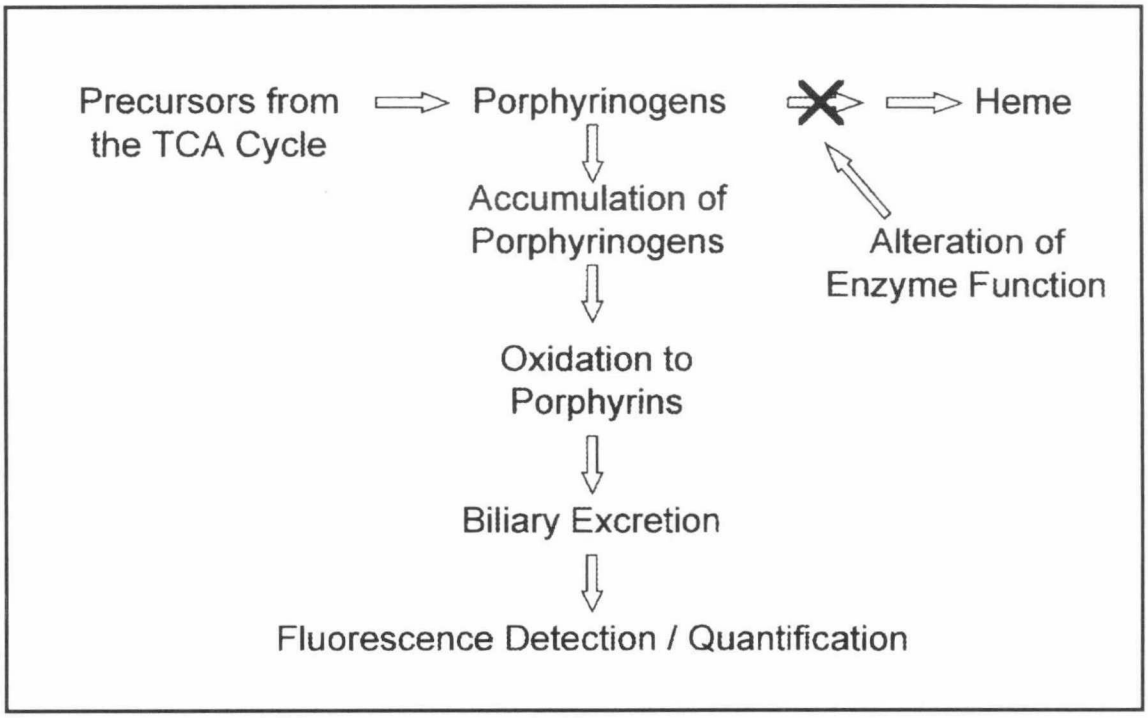


Figure 1.2. Alteration of the heme biosynthetic pathway. Accumulation of heme precursors called porphyrinogens leads to biliary excretion of porphyrins, which may be detected in fecal extracts with fluorescence detection.

range of organic chemicals and heavy metals (Daniell et al., 1997). Changes in porphyrin excretion have also been used as a biomarker in wildlife toxicology studies (Koss et al., 1986; Kennedy and Fox, 1990; Brock, 1992; Akins et al., 1993; Chamberland et al., 1995; Fossi et al., 1997a, b). Total porphyrin levels in fecal samples were also elevated in river otters inhabiting oiled areas of Prince William Sound compared with nonoiled areas following the oil spill (Blajeski et al., 1996). The purpose of this thesis research project was to evaluate the use of the entire profile of porphyrins in fecal samples of river otters as a biomarker of oil exposure.

The research objectives of this project were divided into five analytical phases: 1.) Develop a rapid and efficient method for characterizing the profile of porphyrins in a large number of fecal samples from river otters; 2.) Compare high-performance liquid chromatography and spectrofluorometric methods for the analysis of porphyrins fecal samples; 3.) Characterize the profile of porphyrins in fecal samples collected from oiled and nonoiled areas within Prince William Sound during 1990 and 1996 to determine if differences existed between oiled and nonoiled sites during both years and to determine if recovery had taken place between years; 4.) Characterize the profile of porphyrins in fecal samples of river otters collected at a reference location in southeast Alaska to help establish a reliable baseline for porphyrin excretion in fecal samples of river otters; 5.) Evaluate changes in porphyrin excretion in fecal samples of river otters experimentally dosed with weathered crude oil and compare those changes with those observed in the field studies. These objectives were designed to test the working hypotheses that alterations in heme metabolism occur in river otters as a result of the effects of crude oil exposure and that such alterations may be quantitatively assessed through the measurement of changes in the profile of fecal porphyrins with rapid analytical techniques.

DEVELOPMENT OF EXTRACTION AND HPLC METHODS FOR THE QUANTIFICATION OF PORPHYRINS IN FECAL SAMPLES OF RIVER OTTERS

ABSTRACT

An analytical method to facilitate the rapid extraction and characterization of porphyrins in fecal samples of river otters was developed. Porphyrins were extracted from fecal samples of river otters with hydrochloric acid and concentrated on disposable solid phase extraction cartridges. Separation of porphyrins was accomplished with high-performance liquid chromatography incorporating a 6-minute run time and 5-minute system re-equilibration. Porphyrins were detected with a single monochromator equipped with a 405 nm excitation cut-off filter and a 620 nm emission interference filter with a 10 nm bandwidth. The narrow bandwidth emission filter allowed discrimination of the fluorescence signals of porphyrins from other background fluorescence inherent in the sample. The narrow bandwidth did however decrease the detection sensitivity and necessitated the use of solid phase extraction during the extraction procedure for concentration of porphyrins. The detection limit for porphyrins was approximately 0.01 nmol/g dry fecal material. The results of this research describe a rapid analytical method for detection and quantification of porphyrins in fecal samples of river otters.

2.1 INTRODUCTION

Current analytical methods designed to separate and quantify concentrations of individual porphyrins in biological samples incorporate HPLC separation and fluorescence detection systems (Lim, 1991). The trend in refinement of these methods has been to shorten the separation time and increase the sensitivity and selectivity of detection of individual porphyrins. The primary goal of the HPLC method development of this project was to optimize separation and detection conditions to facilitate rapid and efficient quantification of porphyrins in river otter fecal samples.

Porphyrins have diagnostic fluorescence spectra which allows their sensitive identification with current fluoresence detection systems capable of detecting concentrations of porphyrins in the nanomolar to picomolar range (DeMatteis and Lim, 1994). Current fluorescence detection systems employing a series of photomultiplier tubes and variable wavelength scanning capability facilitate increased selectivity and sensitivity through amplification of the fluorescence signal at specific wavelengths (McPherson Corp., 1998, Personal communication). Combining HPLC separation and fluorescence detection allows individual porphyrins with characteristic retention times to be identified and quantified in biological samples.

Early separation of porphyrins with HPLC involved normal phase separation of porphyrin methyl esters. More recently, reverse phase chromatographic systems employing octadecylsilyl (C-18) -bonded chromatography columns have been used to separate porphyrin free acids, eliminating the need for derivatization of porphyrin methyl esters (Lim, 1991). Separation of both type I and type III porphyrin isomers from urine and fecal samples with a forty minute gradient elution in a reverse phase system has been described (Lim and Peters, 1984). Alternatively, a five minute gradient elution has been reported for separation of porphyrins in tissue samples with a sodium phosphate-methanol mobile phase (Kennedy et al., 1986). Also, a seven minute gradient elution for separation of

porphyrins in fecal samples with an ammonium acetate-methanol mobile phase has been described (Perkins, S., 1999; personal communication).

The chromatographic conditions used in this project were developed for rapid determination of porphyrins in a large number of samples. The specific fluorescence detection parameters described in this project reflected the need to detect porphyrins in a complex sample matrix while restricted to modifications of existing equipment. The sequence of events describing the HPLC method development began with modification of the fluorescence detector to improve the selectivity and sensitivity of the detection of porphyrins in the fecal matrix. Different chromatographic conditions designed to facilitate rapid separation of porphyrins were then tested through modifications of the solvent system, run time, and gradient elution components of procedures described in the current literature.

2.2 METHODS

Sample Collection and Preparation

River otter fecal samples representing field collections at oiled and nonoiled sites within Prince William Sound during 1990 and 1996 and stored frozen were obtained from the archive at the Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, Alaska, USA. The 1990 year class samples were collected at Knight Island (oiled area; 60°30'N, 147°40'W; n = 22) and Esther Passage (nonoiled area; 60°53N, 147°55'W; n = 28). The 1996 year class samples were collected at the same oiled area at Knight Island (n= 23) and Jackpot Bay (nonoiled area; 60°25N, 147°30'W; n = 23). Fecal samples were collected from river otters involved in an oil dosing experiment at the Alaska Sea Life Center, Seward, Alaska. Fecal samples collected from the experimental otters were placed in plastic Whirl-Pak® bags and stored frozen. Fecal samples also were collected from latrine sites identified in northern Lynn Canal, Alaska during June and July 1998. Those fecal samples were placed in plastic bags and stored frozen at -70°C until analysis.

Wet Weight-DryWeight Analysis

To determine the potential effect of variability in moisture content of fecal samples on quantification of porphyrins, a wet weight-dry weight analysis was conducted. Six fecal samples were weighed on a bench-top Mettler balance. The fecal samples were lyophilized for 24 h, weighed, lyophilized for a second 24 h and re-weighed. The percent difference in sample weight was calculated after each 24 h lyophilizing session. All fecal samples representing field collections and the dosing experiment were weighed before and after 24 h of lyophilizing. The differences in weights obtained before and after lyophilizing were used to evaluate the potential effect of moisture content on quantification of concentrations of porphyrins.

Reagents

ACS-grade hydrochloric acid, sodium phosphate, acetonitrile and acetone were used for porphyrin extraction from the fecal samples. ACS-grade ammonium acetate, and HPLC-grade methanol were used for HPLC analysis. Milli Q-18 water was used for all solvent preparations.

Standards

The porphyrin standards used for determination and quantification of fecal porphyrins included a standard chromatographic marker kit containing the number I isomers of 8, 7, 6, 5, 4 carboxylate porphyrins and mesoporphyrin IX, as well as coproporphyrin III, deuteroporphyrin IX and protoporphyrin IX. All porphyrin standards were purchased from Porphyrin Products, Logan, Utah, USA.

Chromatographic Conditions

The HPLC system used to separate porphyrins consisted of two Waters 510 pumps (Waters Corp., Milford, MA, USA), a Rheodyne 7125 manual injector valve equipped with a 5 µl injection loop (Rheodyne Corp., Cotati, CA, USA) and a 3 by 0.46

cm Luna C-18 column equipped with a Security Guard® cartridge system (Phenomenex Corp., Torrance, CA, USA). Baseline 810 software (Waters Corp.) was used for system control.

Fluorescence detection was facilitated by a McPherson FL-748, single-monochromator fluorescence detector (McPherson Corp., Chelmsford, MA, USA). The detector was equipped with a 405 nm excitation cut-off filter. Optimization of the fluorescence detection system was accomplished with the installation of a narrow-band interference emission filter and a red-sensitive photomultiplier tube.

Development of the HPLC method involved testing modifications of procedures described by Kennedy and James (1993) and Perkins, S., (1998 Personal communication). Adjustments were made to the slope and duration of each time component of the two-part mobile phase gradient until all porphyrins were detected on the chromatogram and porphyrin peaks were sharp and optimally spaced (Bidlingmeyer, 1992). Adjustments to the gradient were confined to a 6 min total run time in keeping with the objective to develop a method of porphyrin separation and detection that was as rapid as possible.

Porphyrin Extraction and Concentration

Porphyrin extraction and solid phase extraction (SPE) concentration procedures were developed by testing modifications of procedures described by Bowers et al. (1992) and Kennedy and James (1993). Several different sizes of SPE columns were tested to determine the appropriate size necessary for accommodating the volume and matrix characteristics of the fecal extracts. To determine the appropriate solvent systems for extraction and SPE concentration, aliquots of known concentrations of porphyrin standards were processed through different modifications of extraction and SPE concentration procedures. Fecal samples spiked with aliquots of porphyrin standards were processed through different modifications of extraction and SPE concentration procedures to determine the optimum solvent systems for separation of porphyrins from unwanted components of the fecal matrix and to determine the efficiency of the extraction and SPE

concentration procedures.

Attempts were made to estimate the quantity of porphyrins lost through the field samples extraction procedure by spiking fecal samples with known concentrations of porphyrins prior to processing the samples through the extraction procedure. To estimate the extraction efficiency of the procedure used for the 1990 and 1996 year class field samples, aliquots of a 3.0 μM chromatographic marker kit containing 9 nmol, 6 nmol, and 3 nmol of porphyrins were added to three 1.0 g portions of two different fecal samples in which no porphyrins were detected with prior HPLC analysis. For comparison, methods standards were also run in which solutions of porphyrin standards were processed through the extraction procedure. The number of nanomoles of each porphyrin were calculated with a seven-point calibration curve and compared to the actual number of nanomoles added to the sample. To compare the extraction efficiency of the two different procedures used for the field samples and dosing experiment samples, two fecal samples representing the 1996 reference area were extracted with the method used for the dosing experiment samples and the results were compared to those obtained previously with the field samples method.

Porphyrin Analysis

For HPLC analysis, the dry sample residues containing the porphyrins were thawed and reconstituted in 6.0 N HCl. The dry residues from the 1990 and 1996 field samples were reconstituted in 500 uL of 6.0 N HCl, while the dry residues from the samples collected during the dosing experiment and from Lynn Canal were reconstituted in 100 ul of 6.0 N HCl. Reconstituted supernatants were injected directly into the HPLC system. Attempts to clarify the supernatants through filtration with 0.45μm Millex-HV filters were unsuccessful. The Security Guard cartridge system was therefore relied upon to prevent deterioration of the analytic column. The fecal supernatants were run on the HPLC in ten-sample sequences, preceded by and followed by injection of a 3.0 μM chromatographic porphyrin marker standard. The Security Guard cartridge was replaced after each ten-

sample sequence. To evaluate porphyrin loss due to potential deterioration of the Security Guard cartridge over the 10-sample analysis sequence and to evaluate the average interassay variation, the percent difference in concentrations of the porphyrin standards run before and after each 10-sample sequence was calculated.

Chromatograms for each sample and standard run were saved on floppy disk, transferred to a Maxima 820 chromatographic software program (Waters Corp.) on a separate computer and smoothed with the Chromatogram Operations function. The default smoothing setting of 13 convolution points was used. The smoothed chromatograms were transferred back to Baseline 810 for peak integration. All peaks for the sample chromatograms were identified by comparing the retention times of the sample peaks to those of standard peaks. All peak baselines were drawn manually. The chromatograms with identified peaks and baselines were re-processed with Baseline 810 for peak integration to obtain the peak areas used for quantitative analysis.

Porphyrin Quantification

The use of two different internal standards was investigated: 2,4-diglycol-deuteroporphyrin (Beukeveld et al., 1987), and 2-Vinyl-4-hydroxymethyldeuteroporphyrin IX (Carlson et al. 1984). The stability of both porphyrins in the solvent systems developed for the extraction and SPE concentration procedures was tested by dissolving the porphyrins in the solvents and comparing their fluorescence signals over time by repeated analysis with HPLC. Fluorescence signals obtained on successive runs were compared to the fluorescence signals obtained upon first mixing of the porphyrin in the solvents, considered time zero.

Interassay Variation

The variation between each sample analysis series due to fluctuations in HPLC system efficiency was evaluated by running porphyrin standards before and after each sample analysis series. Interassay variation was then determined by calculating the percent

difference in concentrations of the porphyrin standards run before and after each sample series.

2.3 RESULTS

Wet Weight-Dry Weight Analysis

The river otter fecal samples varied considerably in moisture content (Table 2.1 a). The average percent difference after 24 h of lyophilizing was 35.9% with a standard deviation of 21.2 and a percent coefficient of variation of 59.0%. After 48 h of lyophilizing, the average percent difference was 1.45% with a corresponding standard deviation of 0.69 and percent coefficient of variation of 47.4% (Table 2.1 b). After 24 h, all of the fecal samples were visibly dry. The average percent differences in the weight of all of the fecal samples representing the 1990 and 1996 year classes ranged from 9.05% to 18.1% (Table 2.2). The corresponding percent coefficients of variation for the differences in fecal weights ranged from 10.6% to 91.2%. The average percent difference

Table 2.1. Differences in weights of six river otter fecal samples after 24 h (a) 48 h (b) of lyophilizing.

Time	Mean (% Difference)	Standard Deviation	% Coefficient of Variation [a]	Range (% Difference)
a. 24 Hours	35.9	21.2	59.0	13.8 - 57.8
b. 48 Hours	1.5	0.7	47.4	0.8 - 2.4

[a] % Coefficient of Variation = (Mean/Standard Deviation) x 100
n = 6

Table 2.2. Differences in weights of river otter fecal samples representing the 1990 and 1996 year classes after 24 h of lyophilizing.

Sample Series	% Difference (Mean ± Standard Deviation)	% Coefficient of Variation [a]	Range (% Difference)
Knight Island 1990 (n = 22)	17.8 ± 4.0	22.3	1.1 - 42.0
Esther Passage 1990 (n = 28)	18.1 ± 1.9	10.6	1.7 - 47.2
Knight Island 1996 (n = 23)	9.1 ± 6.3	70.1	1.3 - 24.5
Jackpot Bay 1996 (n = 23)	13.5 ± 12.3	91.2	2.3 - 57.9
Dosing Experiment (n = 165)	32.7 ± 9.2	28.1	4.6 - 48.6

[a] % Coefficient of Variation = (Mean/Standard Deviation) x 100

in weight of the fecal samples collected during the dosing experiment after 24 h of lyophilizing was 32.7% with a percent coefficient of variation of 28.1%.

The concentration of porphyrins in each fecal extract was calculated with a seven-point calibration curve (0.0 μM to 3.0 μM) developed with solutions of porphyrin standards dissolved in 6 N HCl. The concentration of porphyrins were reported as nanomoles of porphyrins per gram of dry fecal material.

Chromatographic Conditions

A two-component mobile phase consisting of ammonium acetate (1.0 M, pH 5.16) as solvent A and 100% methanol as solvent B was adopted for porphyrin separation. The use of sodium phosphate (0.1 M, pH 3.5) was tested as solvent A, but was replaced with ammonium acetate to reduce salt formation and deterioration of the pump-head seals. The ammonium acetate buffer also facilitated greater separation of the eight through four carboxylate porphyrins. The pK_a's of eight and four carboxylate porphyrins are 7.3 and 6.8, respectively, (Perkins and Johnson, 1989) and therefore, increasing the pH from 3.5 (sodium phosphate) to 5.16 (ammonium acetate) may have allowed earlier removal of the higher-carboxylated porphyrins from the column to facilitate greater separation of the porphyrins on the chromatogram. Elution commenced upon injection and proceeded from 25% B at time zero to 50% at 1.0 min, then to 95% B at 4.0 min, remained at 95% B for 1.5 min and returned to 25% B at 6 min. The column was allowed to re-equilibrate for 5 min at 25% B before subsequent injections.

Fluorescence Detection

Initial attempts to detect porphyrins in the fecal samples with the detector equipped with a 440 nm wide-band emission cut-off filter resulted in the detection of fluorescing components of the fecal matrix, which obscured the chromatogram and made detection of porphyrins impossible (Fig. 2.1). Chamberland et al. (1995) reported a similar problem of background fluorescence in mussel extracts obscuring the chromatogram and recommended the use of a narrow-band grating holographic monochromator to reduce the background fluorescence and allow quantification of individual porphyrins. This was essentially simulated with the fluorescence detector in our laboratory with the installation of a 620 nm emission filter with a 10 nm band width. This narrow-band, glass emission filter allowed the detection of the fluorescence signal representing the approximate center of the emission spectra of porphyrins and eliminated the detection of interfering fluorescing components of the fecal matrix (Fig. 2.2). The installation of the narrow-band, 620 nm emission filter did, however, decrease the intensity of the fluorescence signal considerably and necessitated the installation of a new red sensitive photomultiplier tube to enhance the detection of porphyrins at low concentrations.

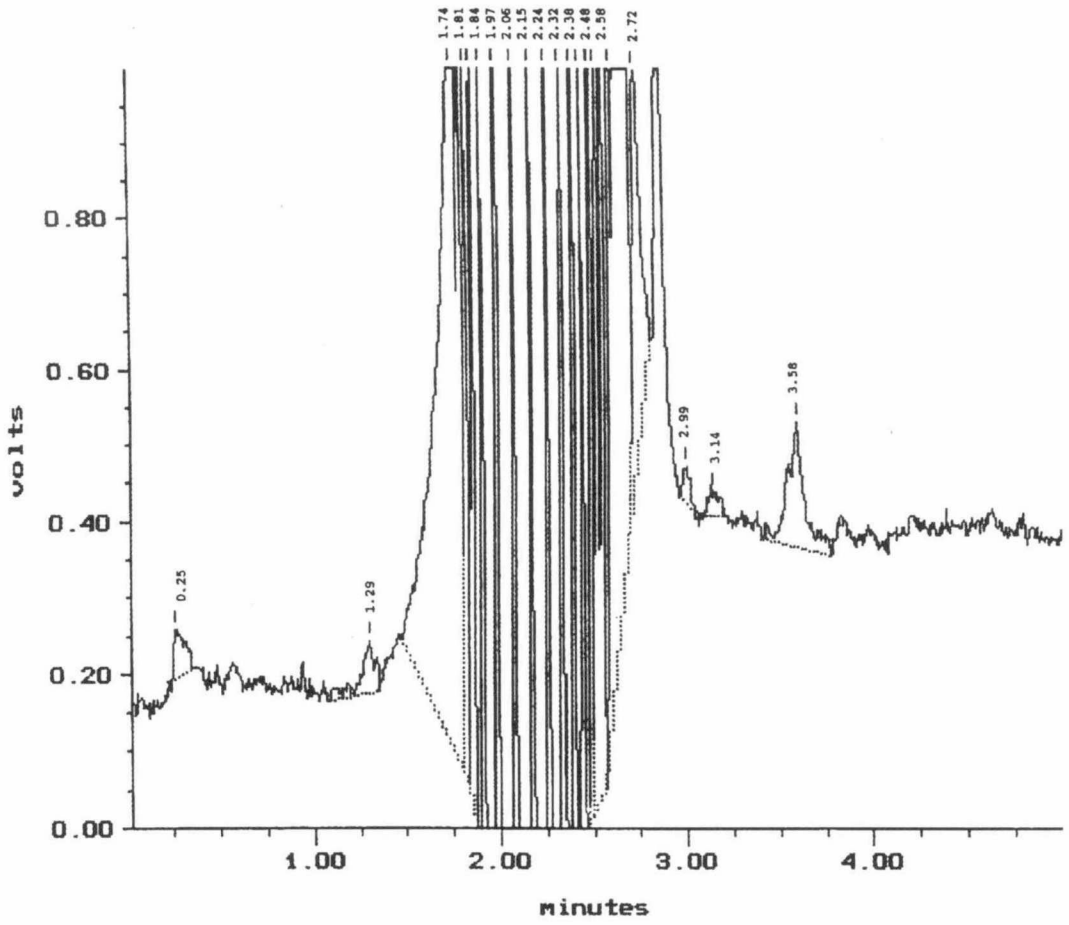


Figure 2.1. Chromatogram of a fecal extract from a river otter obtained with a 440 nm emission cut-off filter. The extract was spiked with an aliquot of a standard chromatographic marker kit containing the 8, 7, 6, 5, 4, carboxylate porphyrins and mesoporphyrin. Background fluorescing compounds inherent in the fecal matrix obscure the detection of the 8 through 4 carboxylate porphyrins.

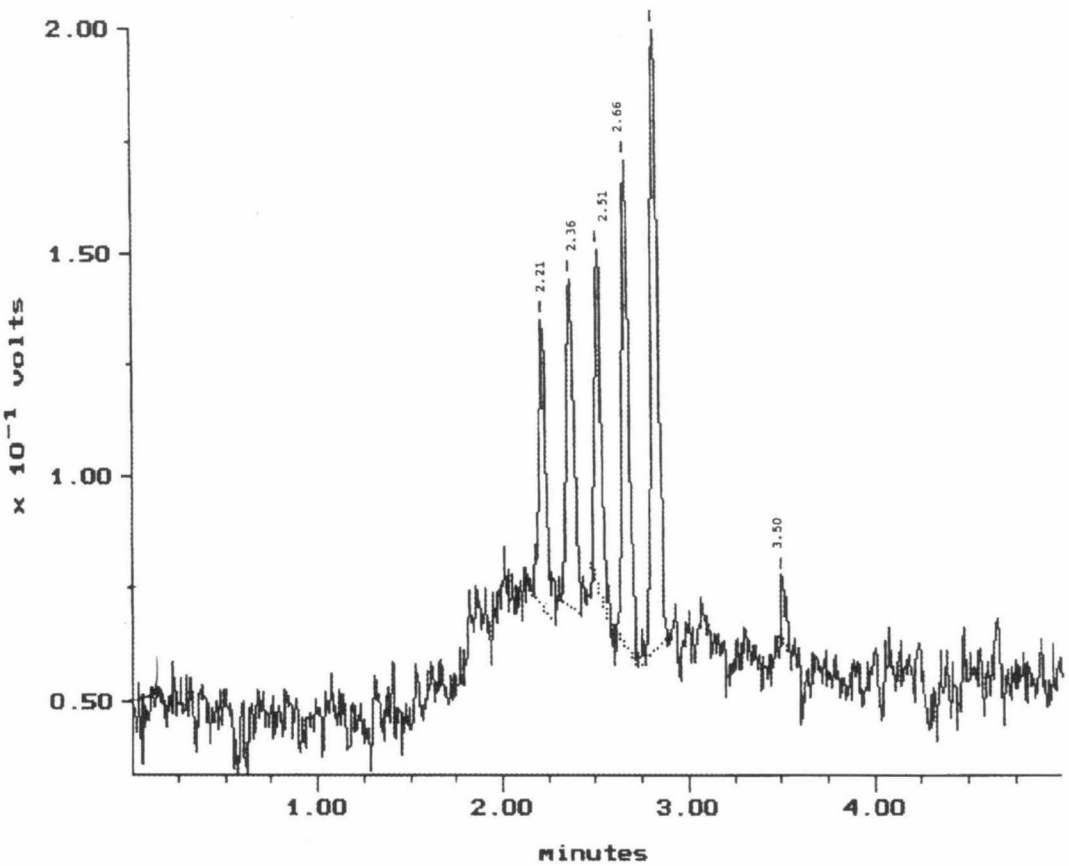


Figure 2.2. Chromatogram of a fecal extract from a river otter obtained with a 620 nm interference emission filter with a 10 nm band width. The extract was spiked with an aliquot of a chromatographic marker kit containing the 8, 7, 6, 5, 4 carboxylate porphyrins and mesoporphyrin. Note that background fluorescence inherent in the fecal sample is significantly reduced allowing discrimination of the individual porphyrins.

Detection Limit

The detection limit for porphyrin standards under the detection conditions used in this project was approximately 500 nmol/5µl injection. The detection limit for porphyrins in the fecal sample extracts was 0.01nmol/g dry fecal weight. The eight through four carboxylate porphyrins were separated by approximately 0.41 min within the 6 min run time.

Porphyrin Extraction and Concentration

Porphyrin extraction and isolation from the fecal samples were accomplished with a modification of a procedure described by Bowers et al. (1992). Porphyrins were extracted with HCl, concentrated on SPE cartridges (Sep-Pak®, Waters. Corp.) and eluted into cryogenic tubes with a system of polar solvents that was evaporated to produce a dry residue containing the porphyrins.

To extract porphyrins from the fecal samples representing the 1990 and 1996 year classes, the following procedure was used. Five milliliters of 6.0 N HCl was added to approximately 1.0 g of dry fecal material and the sample was macerated with a glass rod for approximately 1 min. The sample was mixed with a bench top vortex for 1.0 min, sonicated in a water bath for 5.0 min, and mixed again for 1.0 min. Five milliliters of sodium phosphate buffer (0.01 M, pH 3.5) was then added to the sample. The sample was mixed again for 1.0 min and centrifuged for 10.0 min at 4,000 rpm. The coarse pellet was removed from the centrifuge tube and the sample was centrifuged again for 10.0 min at 4,000 rpm.

The Sep-Paks® were attached to a Supelco Visiprep vacuum manifold (Supelco Corp., Bellefonte, PA, USA) and prepared by washing first with 7.0 ml of acetonitrile followed by 7.0 ml of sodium phosphate buffer (0.01 M, pH 3.5). Eight milliliters of the fecal supernatant was delivered to the Sep Paks® and allowed to gravity feed. The Sep-Paks® were washed with 3.0 ml of sodium phosphate buffer (0.01 m, pH 3.5) to facilitate complete delivery of the supernatant to the Sep Paks®, followed by 7.0 ml of sodium

phosphate buffer (0.01M, pH 7.5) to enhance recovery of the porphyrins. The concentrate containing the porphyrins at the top of the Sep-Paks® was eluted into 5.0 ml cryogenic centrifuge tubes with 1.0 ml of acetonitrile, followed by 0.5 ml of acetonitrile : 1.0 N HCl (1:1, v/v), and finally 1.0 ml of acetone under vacuum. The tubes containing the eluates were placed in a water bath at 55°C and evaporated to a dry residue under a stream of air. The dry residues containing the porphyrins were stored frozen at -70°C.

Supernatants of fecal samples collected from the experimental river otters and the fecal samples collected in the field in Lynn Canal during summer 1998 proved to be more difficult to clarify than the those from the 1990 and 1996 year class samples, indicating that some deterioration of the 1990 and 1996 samples may have occurred. The following modifications were made to the extraction procedure used for the 1990 and 1996 samples in an attempt to improve clarification of the supernatants. Five milliliters of Milli-Q 18 water replaced the sodium phosphate buffer added to acidic fecal extract. Removal of coarse pellet was not found to be necessary and this step was omitted. The second centrifugation step was replaced with microcentrifugation of 2 ml aliquots of each fecal supernatant at 7,000 rpm. The Sep-Pak® concentration procedure was modified by the addition of 1.6 ml, rather than 8 ml, of the final supernatant.

Comparison of Field Sample and Dosing Experiment Extraction Procedures

The addition of a microcentrifuge step to the extraction procedure used for the fecal samples collected during the dosing experiment and the fecal samples collected in Lynn Canal did not result in a substantial loss of porphyrins (Table 2.3). With the exception of Uro I for the JPLSJ2 sample, porphyrin recoveries were higher for the dosing experiment procedure than for the field procedure.

Table 2.3. Comparison of porphyrin recoveries for two different procedures to extract porphyrins from river otter fecal samples.

Sample	Porphyrin	Field Procedure (nmol/g)	Dosing Experiment Procedure (nmol/g)
JPLSJ2	Uro I	0.26	0.21
JPLSJ2	Hepta I	0	0.20
JPLSJ2	Hexa I	0	0
JPLSJ2	Penta I	0	0
JPLSJ2	Copro I	0	0
JPLSJ2	Copro III	0	0.33
JPLS11	Uro I	0.34	0.44
JPLS11	Hepta I	0	0.23
JPLS11	Hexa I	0	0
JPLS11	Penta I	0	0
JPLS11	Copro I	0.35	0.60
JPLS11	Copro III	0.60	0.85

Extraction Efficiency with the Field Sample Extraction Procedure

The average extraction efficiencies for all porphyrins of the chromatographic marker kit for the two methods standards trials were 61% and 89% (Table 2.4 a). The average extraction efficiency for all porphyrins of the chromatographic marker kit reported as a percentage of porphyrins added to the fecal samples (sample spikes) ranged from 54% to 124% (Table 2.4 b). Percent recoveries for individual porphyrins for each sample were also variable. The chromatography for the extraction efficiency samples was, on average, very poor with a large hump in the baseline occurring in the eight through four carboxylate porphyrin region, and with peaks appearing broad and poorly defined. Also, peaks not observed with previous HPLC analysis, were present and had retention times different from the retention times of the porphyrin standards.

Table 2.4. Extraction efficiencies for the field sample extraction procedure.

a.

Trial	Porphyrin	Nanomoles Applied [a]	Nanomoles Recovered	Average % Recovery
Method Standard 1	Uro I	1	0.67	67
Method Standard 1	Hepta I	1	0.57	57
Method Standard 1	Hexa I	1	0.68	68
Method Standard 1	Penta I	1	0.54	54
Method Standard 1	Copro I	1	0.61	61
Method Standard 2	Uro I	1	0.86	86
Method Standard 2	Hepta I	1	0.82	82
Method Standard 2	Hexa I	1	0.95	95
Method Standard 2	Penta I	1	0.93	93
Method Standard 2	Copro I	1	0.90	90

[a] Nanomoles of porphyrins in an aliquot of 6.0 N HCl treated as a sample and processed through the extraction procedure.

b.

Sample	Spike (nmol) [a]	Nanomoles Recovered					Average % Recovery
		Uro I	Hepta I	Hexa I	Penta I	Copro I	
HBL11	9	0.53	0.58	0.62	0.47	0.25	54
HBL11	6	0.48	0.71	0.49	0.50	0.71	97
HBL11	3	0.16	0.60	0.43	0.23	0.41	124
HBL20	9	0.71	0.78	0.76	0.76	0.93	88
HBL20	6	0.38	0.71	0.45	0.34	0.14	67
HBL20	3	0.21	0.53	0.31	0.27	0.43	116

[a] Nanomoles of porphyrins in 6.0 N HCl solution added to each 1.0 g portion of fecal sample prior to processing through the extraction procedure.

Internal Standard Assessment

The use of an internal standard described by Beukeveld et al. (1987), 2,4-diglycoldeuteroporphyrin, was tested. Although this porphyrin dissolved readily and was stable in 6 N HCl, it had a retention time nearly identical to Hepta I under the chromatographic conditions used in this project and was determined to be inappropriate.

The internal standard, 2-Vinyl-4-hydroxymethyldeuteroporphyrin IX, developed by Carlson et al. (1984) for clinical analysis of porphyrins was unstable under acidic conditions. This porphyrin was stable in methanol:potassium hydroxide (90:10, v/v), yet precipitated out of solution readily when dissolved in 6 N HCl. Within 1 h of addition of

the methanolic solution of the internal standard to 6 N HCl, the porphyrin deteriorated to 4 separate peaks with the three peaks additional to the internal standard peak increasing in size over time (Figs. 2.3 a, b, c).

Inter-assay Variation Between and Within Sample Series

The inter-assay variation between and within each ten sample analysis series for the field samples and dosing experiment samples analyses ranged from 7.29% to 15.8% (Table 2.5). The corresponding percent coefficients of variation ranged from 54.7% to 92.8%.

Table 2.5. Differences between calculated concentrations of porphyrins run before and after each ten-sample HPLC analysis series.

Field Samples	Uro I	Hepta I	Hexa I	Penta I	Copro I
Mean Percent Difference:	9.94	11.28	7.29	8.61	10.76
Standard Deviation:	8.13	6.17	4.21	6.81	9.99
% Coefficient of Variation:	81.81	54.69	57.69	79.06	92.82
Dosing Experiment Samples					
Mean Percent Difference:	10.67	8.80	13.61	9.30	15.79
Standard Deviation:	7.16	5.32	9.50	8.22	12.76
% Coefficient of Variation:	67.14	60.44	69.77	88.33	80.83

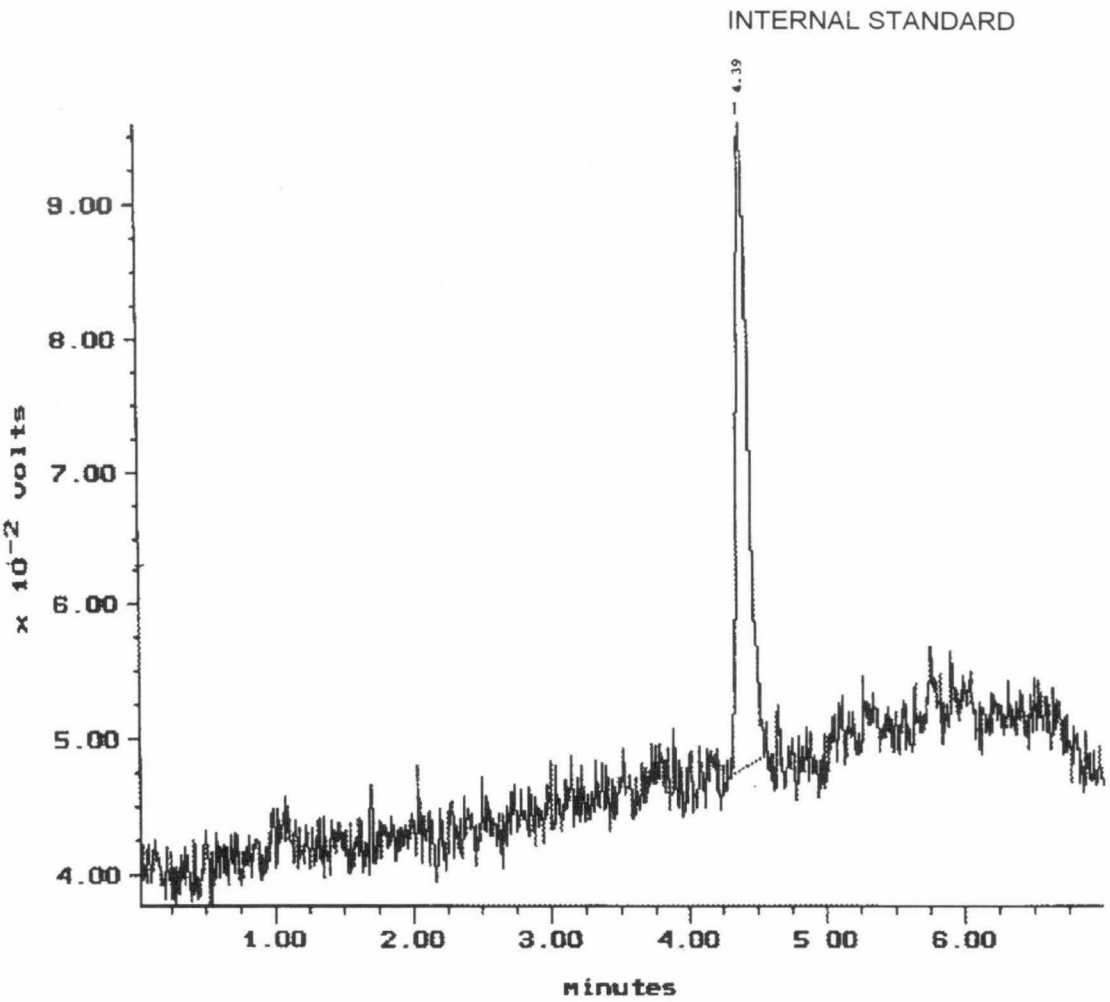


Figure 2.3 a. Chromatogram of the 2-Vinyl-4-hydroxymethyldeuteroporphyrin IX internal standard immediately following reconstitution in 6 N hydrochloric acid.

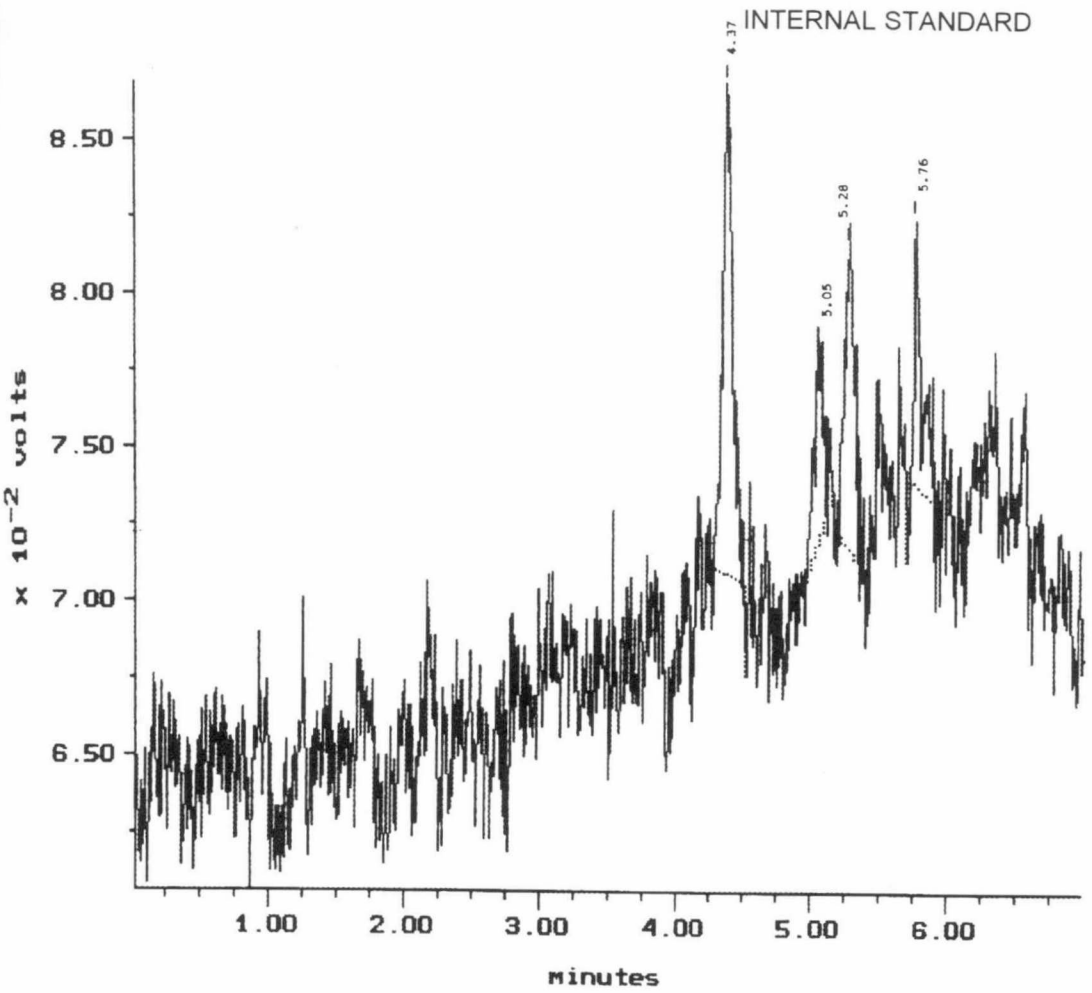


Figure 2.3 b. Chromatogram of the 2-Vinyl-4-hydroxymethyldeuteroporphyrin IX internal standard 1 h following reconstitution in 6 N hydrochloric acid.

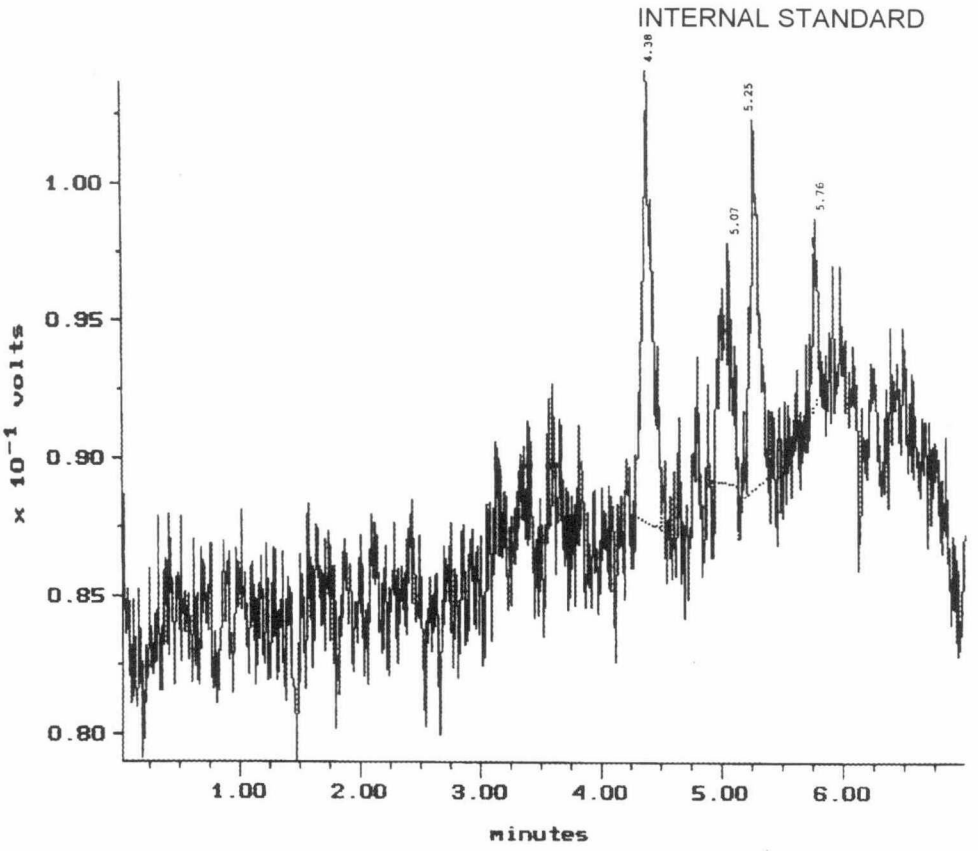


Figure 2.3 c. Chromatogram of the 2-Vinyl-4-hydroxymethyldeuteroporphyrin IX internal standard 2 h following reconstitution in 6 N hydrochloric acid.

2.4 DISCUSSION

To quantify fecal porphyrin excretion, values for porphyrins detected by HPLC were adjusted by the dry weight of the portion of the fecal sample used for analysis. The results of the dry weight-wet weight analysis indicate that a high degree of variability in moisture content exists in river otter fecal samples which could substantially effect the accuracy of the calculation of porphyrin excretion. Although this variability, described by the percent coefficient of variation, was considerably higher in the 1996 year class field samples, a high degree of variability (%C.V. = 28.1%) also existed in the moisture content of fecal samples collected "fresh" during the dosing experiment. These results suggest that field conditions, including weather, and age of the sample, are not the only factors influencing moisture content in fecal samples and to standardize fecal weights for quantitative analysis, all samples, regardless of their apparent age or condition, should be dried.

Solid-phase extraction was used for porphyrin analysis for two reasons: 1) The volume of solvent necessary to digest the dry fecal material and produce a supernatant that could be sufficiently clarified for analysis diluted the porphyrins present in the fecal sample to a concentration that was below the detection limit. Concentration of the fecal sample extracts on the Sep-Paks® facilitated the detection of the porphyrins present in the samples under the detection conditions used in this project; 2) The large number of samples needing to be processed required that the extracted porphyrins be rendered stable until HPLC analysis. Solid-phase extraction allowed the concentrated porphyrins to be eluted with a system of polar solvents that could be subsequently evaporated to produce a dried residue containing the porphyrins. Although solid-phase extraction can be very effective for selectively isolating individual components from complex mixtures, solid-phase extraction was used in this project primarily for sample concentration. Attempts to separate porphyrins from all of the matrix components with the Sep-Paks® were complicated by the fact that the same solvent systems necessary for eluting all of the

porphyrins from the cartridges were also those that facilitated the removal of the fecal matrix components. The fluorescing matrix components present in the final eluate, however, did not interfere with HPLC identification and quantification of porphyrins. The detection limit for porphyrins facilitated by the HPLC equipment and conditions used in this project was higher than those reported in the current literature. This was due primarily to the use of a single monochromator fluorescence detector in combination with a narrow-band, interference emission filter. The use of a more advanced fluorescence detector with multiple monochromators and photomultiplier tubes facilitating enhancement of the fluorescence signal would have improved the detection limit and precluded the use of solid phase extraction for porphyrin concentration.

The addition of a microcentrifugation step to the extraction procedure to facilitate the clarification of the extracts from fecal samples collected from the dosing experiment did not result in substantial porphyrin loss. Differences in porphyrin recoveries between the two extraction procedures may have been due to lack of homogeneity of the fecal samples. Sample homogeneity tests were not conducted on the 1990 and 1996 year class field samples, however, because, for most of the samples, the entire fecal sample was not available as a result of deterioration and previous handling.

Given the complexity of the extraction procedure and the potential for porphyrin loss through the centrifugation, SPE concentration, and drying and reconstitution steps, an attempt was made to estimate the amount of porphyrin loss through extraction efficiency tests. The calculated extraction efficiencies varied considerably for each porphyrin within samples as well as overall between samples. These values are suspect, however, due to the poor chromatography and consequential difficulty in quantifying individual porphyrins. The porphyrin standards composing the chromatographic marker kit used for the sample spikes are in the dihydrochloride form, with a chlorine ion positioned on each side of the porphyrin ring. To evaluate if interaction between the porphyrin standards and components of the fecal matrix contributed to the poor chromatography, the porphyrin standards were diluted with fecal extracts and run on the HPLC. Dilution of the porphyrin

standards with the fecal extracts did not result in the deterioration of the chromatography or loss of porphyrins. Alternatively, inconsistent recovery of the dihydrochloride porphyrin standards through the extraction procedure may have been associated with the drying and reconstitution steps, particularly considering that poor recovery was also observed for the methods standards tests. The chromatography for the fecal sample extracts without spiking was good, characterized by sharp, well defined peaks, and was similar to the chromatography of the porphyrin standards not run through the extraction procedure.

The use of an internal standard to quantify porphyrin recovery through the extraction procedure was investigated in light of the potential for loss of porphyrins through the extraction and SPE concentration procedures. Two porphyrins described in the literature for use as an internal standard for porphyrin quantification were tested. 2,4-Diglycoldeuteroporphyrin was found to have a similar retention time as Hepta I under the HPLC conditions used in this project and was determined to be not appropriate. 2-Vinyl-4-hydroxymethyldeuteroporphyrin IX, developed for use as an internal standard for urinary porphyrin analysis, was not stable in 6.0 N HCl, which was used for porphyrin extraction and HPLC analysis in this project. Alternatively, a seven-point calibration curve was developed from a series of external standards for porphyrin quantification.

The variation in porphyrin standard concentrations calculated before and after each sample series, for all porphyrins in all sample series, was within 16%. Factors that may have contributed to the interassay variation include variability in the manual injection system and variability in the solvent pumping system.

Although the primary objective for the development of the methods to quantify porphyrins in the river otter fecal samples was to establish a procedure that was rapid and efficient, the effectiveness of the procedures established was limited by the equipment available in the laboratory. The use of a more advanced fluorescence detector designed to amplify the fluorescence signal would have resulted in a lower detection limit as well as allowed the elimination of the solid phase extraction procedure necessary for concentrating the porphyrins. Elimination of the solid phase extraction step would have

resulted in a considerable time savings as well as a reduction in the inherent variability in porphyrin recovery. Also, the use of an autosampling system may have reduced the interassay variation inherent in the manual injection system.

COMPARISON OF SPECTROFLUOROMETRIC AND HPLC METHODS FOR THE CHARACTERIZATION OF FECAL PORPHYRINS IN RIVER OTTERS

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Running Head: HPLC - Spectrofluorometric Comparison

ABSTRACT

A spectrofluorometric method developed by Grandchamp et al. (1980) for the determination of the ratios of Uroporphyrin I (Uro I), Coproporphyrin III (Copro III), and Protoporphyrin IX (Proto IX) in skin fibroblasts was compared with a high-performance liquid chromatography (HPLC) method for the analysis of porphyrins in fecal samples of river otters (*Lutra canadensis*). Heptacarboxylate porphyrin I (Hepta I) and Coproporphyrin I (Copro I), two porphyrins determined to be critical in defining the porphyrin profile in faecal samples of river otters with the HPLC method, contributed substantially to the calculation of the concentrations of Uro I and Copro III, respectively, in standard solutions of porphyrins with the spectrofluorometric method. Fluorescing components of the fecal matrix complicated the determination of the concentrations of Uro I, Copro III and Proto IX with the spectrofluorometric method and resulted in erroneous values for the concentrations of these porphyrins compared with values determined with the HPLC method. These results indicate that the complexity of the sample, particularly with regard to the potential presence of interfering fluorescing compounds as well as porphyrins additional to Uro I, Copro III and Proto, should be considered prior to the application of the spectrofluorometric method. An alternative HPLC method developed for the rapid characterization of porphyrin profiles in fecal samples of river otters is described.

Keywords: biomarker, spectrofluorometric method, HPLC, fecal porphyrin profiles, river otters, *Lutra canadensis*

3.1 INTRODUCTION

The use of changes in the profile of fecal porphyrins as a biomarker of the effects of crude oil exposure in river otters (*Lutra canadensis*) following the *Exxon Valdez* oil spill in Prince William Sound, Alaska, USA, has been under investigation in our laboratory. River otters occur along shorelines in the Gulf of Alaska and occupy large home ranges within Prince William Sound (Bowyer et al., 1995). Furthermore, river otters, which feed mostly on marine fishes, occupy a critical niche in the coastal ecosystem linking the nutrient dynamics of the aquatic and terrestrial environments (Bowyer et al., 1994; Ben David et al., 1998) and therefore serve as an important indicator species for the evaluation of the chronic effects of exposure to crude oil in biomonitoring and restoration programs following the *Exxon Valdez* oil spill.

Heme biosynthesis and porphyrin excretion have been shown to be sensitive to a number of chemicals in the environment (DeMatteis and Lim, 1994). Elevation of porphyrins in blood, urine, and feces due to exposures to heavy metals and halogenated hydrocarbons has been measured in laboratory animals and humans (Daniell et al., 1997). Porphyrin levels also have been used as an indicator of chemical exposure in studies of wildlife. Elevated levels of highly carboxylated porphyrins occurred in livers of herring gull chicks (*Larus argentatus*) collected from sites within the Great Lakes contaminated with polyhalogenated aromatic hydrocarbons (Kennedy and Fox, 1990). Measurements of total porphyrin levels were higher in fecal samples of river otters inhabiting oiled areas in comparison with nonoiled areas of Prince William Sound, Alaska, USA, following the *Exxon Valdez* oil spill (Blajeski et al., 1996).

Field monitoring programs, designed around the use of changes in the profile of fecal porphyrins as a biomarker of contaminant exposure, involve the collection and analysis of large numbers of fecal samples necessitating the development of analytical methods that are rapid and efficient. The application of a spectrofluorometric method for the rapid determination of the ratios of Uro I, Copro III, and Proto IX in skin fibroblasts

(Grandchamp et al., 1980) has been reported more recently for the evaluation of porphyrin levels in both laboratory and wild marine organisms (Fossi et al., 1996; 1997a,b). The spectrofluorometric method is based on the measurement of the relative fluorescence signals obtained at each of three discriminating pairs of excitation-emission wavelengths to determine the ratios of Uro I, Copro III, and Proto IX present in biological samples. The purpose of this study was to test the effectiveness of the spectrofluorometric method for the characterization of porphyrin profiles in fecal samples of river otters through comparison with results from an HPLC method.

3.2 METHODS

Sample Collection and Preparation

Fecal samples were obtained from the archive at the Institute of Arctic Biology at the University of Alaska Fairbanks, Fairbanks, Alaska, USA. Fecal samples had been collected from field locations within Prince William Sound during 1990, placed in plastic bags and stored in a freezer at -70°C. The samples were lyophilized for 24 h prior to porphyrin extraction and analysis.

Standards

Porphyrin standards used for HPLC analysis included a chromatographic marker kit containing the number I isomers of 8,7,6,5,4 carboxylate porphyrins and mesoporphyrin IX, Copro III, and Proto IX. The porphyrin standards used for the spectrofluorometric analysis included Uro I, Copro III, and Proto IX fluorescence marker kits, Hepta I and Copro I. All porphyrin standards were dissolved in 6N HCl. The concentrations of Hepta I, Copro I, and Proto IX were determined by measuring the absorbance at the Soret peak and applying the appropriate molar absorptivity. All porphyrin standards were purchased from Porphyrin Products, Logan, Utah, USA.

Reagents

Reagents used in extraction procedures included ACS-grade hydrochloric acid, sodium phosphate (monobasic), acetonitrile, and acetone. HPLC reagents included ACS-grade ammonium acetate and HPLC-grade methanol. Milli-Q 18 water was used for all solvent preparations.

Equipment

Porphyrins were concentrated on disposable, 500-mg, trifunctional, C-18 (tC-18) Sep-Pak® cartridges (Waters Corp., Milford, MA, USA). Separation of porphyrins was facilitated by a high-performance liquid chromatography system consisting of two Waters 510 pumps (Waters Corp.), a Rheodyne 7125 injector valve equipped with a 5-uL injection loop (Rheodyne Corp., Cotati, CA, USA) and a 3 by 0.46 cm Luna C-18 column equipped with a Security Guard® cartridge system (Phenomenex Corp., Torrance, CA, USA). Porphyrins were detected with a McPherson FL-748 fluorescence detector equipped with a 405 nm excitation cut-off filter, a 620 nm interference emission filter with a 10 nm bandwidth and a red sensitive photomultiplier tube (McPherson Corp, Chelmsford, MA, USA). Baseline 810 and Maxima 820 chromatographic software (Waters Corp.) were used for system control and peak integration.

Spectrofluorometric analysis was accomplished with an SLM Aminco spectrofluorometer model SPF 500C equipped with an LX 300 UV lamp and a Hamamatsu R928P red sensitive photomultiplier tube. A 1.0 ml quartz cuvette was used for spectrofluorometric measurements.

Porphyrin Extraction

Extraction and isolation of porphyrins from the fecal samples were accomplished with a modification of a procedure described by Bowers et al. (1992). Porphyrins were extracted with HCl and concentrated on trifunctional C-18 Sep-Pak® cartridges (Waters Corp). Five milliliters of 6N HCl were added to approximately 1.0 g of dried fecal material

and the sample was macerated with a glass rod for 1 min. The sample was then mixed with a bench top vortex for 1 min, sonicated in a water bath for 5 min and mixed again for 1min. Five milliliters of sodium phosphate buffer (0.01M, pH 3.5) were added to the sample, which was mixed for 1 min. The sample was then centrifuged for 10 min at 4,000 rpm. Following this step, the coarse pellet was removed and the sample was centrifuged again for 10 min at 4,000 rpm. Eight milliliters of the supernatant were delivered to the Sep-Pak® cartridges.

Trifunctional C-18 Sep-Pak® cartridges were used for porphyrin concentration to avoid stripping of the alkyl chain from the support under acidic conditions. The tC-18 Sep-Paks® were connected to a Supelco Visiprep vacuum manifold (Supelco Corp., Bellefonte, PA, USA) and washed with 7 ml of acetonitrile followed by 7 ml of sodium phosphate buffer (0.01M, pH 3.5). Eight milliliters of fecal supernatant were delivered to the Sep-Paks and allowed to gravity feed. The Sep-Paks® were washed with 3 ml of sodium phosphate buffer (0.01M, pH 3.5) to facilitate complete delivery of the supernatant to the Sep-Pak®, followed by 7 ml of sodium phosphate buffer (0.01M, pH 7.5) to adjust the pH of the column and enhance recovery of porphyrins. Residual sodium phosphate buffer was removed from the column with vacuum set at 5 mmHg. The concentrate at the top of the Sep-Pak® containing the porphyrins was eluted into 5 ml cryogenic centrifuge tubes with 1 ml of acetonitrile, followed by 0.5 ml of acetonitrile : 1.0N hydrochloric acid (1:1, v/v) , followed by 1 ml of acetone under vacuum set at 15 mmHg. The tubes containing the eluates were placed in a water bath at 55°C and evaporated under a stream of air. The dried residues containing the porphyrins were stored at -70°C. For HPLC analysis, the dried residues were reconstituted in 0.5 ml of 6N hydrochloric acid and injected directly into the HPLC system.

HPLC Analysis

The HPLC method used was a modification of a procedure described by Kennedy and James (1993). Separation of porphyrins was facilitated by a 6-min gradient elution and

a two- component mobile phase consisting of ammonium acetate (1.0M, pH 5.16) as solvent A and 100% methanol as solvent B. Gradient elution commenced upon injection at 25% B, increased to 50% B in 1 min, then to 95% B in 3 min, remained at 95% B for 1.5 min, and returned to 25% B in 0.5 min. The column was allowed to re-equilibrate for 5 min at 25% B before the next injection.

The concentrations of porphyrins in each fecal sample were calculated with a seven-point calibration curve ranging from 0.0 μM to 3.0 μM. Calibration standards for porphyrins were dissolved in 6N hydrochloric acid and remained stable for at least 24h.

Spectrofluorometric Analysis

Spectral measurements were made with the excitation bandwidth set at 0.25 nm and the emission bandwidth set at 20 nm. Maintaining a very narrow excitation bandwidth was necessary to discriminate between Uro I and Copro III. The emission bandwidth was set at 20 nm to obtain an adequate fluorescence signal from such a narrow range of excitation energy.

To determine the three pairs of excitation-emission wavelengths necessary to discriminate between Uro I, Copro III, and Proto IX, standard solutions of the three porphyrins were scanned individually at 90 different pairs of excitation-emission wavelengths from 395-595 nm Ex to 410-610 nm Em, and the fluorescence signal for each porphyrin at each wavelength pair was recorded. The choice of the wavelength pairs was based on those that produced the greatest fluorescent signal for each porphyrin of interest relative to the other two porphyrins (Wehry 1981).

To test the accuracy of the spectrofluorometric method, fluorescence signals were measured at each of the three pairs of wavelengths for four different mixtures of Uro I, Copro III, and Proto IX and the relative concentrations of the porphyrins were calculated. The actual concentrations were then compared with the calculated concentrations.

To determine the contribution of Hepta I and Copro I to the calculation of the concentrations of Uro I, Copro III, and Proto IX, Hepta I and Copro I were added to

three different mixtures of porphyrin standards. The concentrations of Uro I, Copro III, and Proto IX were determined with Hepta I and Copro I present in the standard mixtures and compared with the actual concentrations.

The concentrations of Uro I, Copro III, and Proto IX in 10 different fecal extracts were also calculated with the spectrofluorometric method and compared with concentrations calculated with the HPLC method. Fecal extracts prepared for HPLC analysis were diluted 100 fold prior to spectrofluorometric analysis.

Statistical Analysis

A multivariate analysis of variance model (PROC GLM, MANOVA; SAS) was used to evaluate the contribution of the addition of Hepta I and Copro I to the standard porphyrin mixtures on the calculation of the concentrations of Uro I, Copro III, and Proto IX. One-tailed contrasts (PROC GLM, CONTRAST; SAS) were used to evaluate how Hepta I and Copro I contributed to the calculated concentrations of Uro I, Copro III, and Proto IX independently. Differences in concentrations of the porphyrins in the fecal extracts determined with the HPLC and spectrofluorometric methods were evaluated with paired t-tests. Results were determined to be significant at $p < 0.05$. Statistical analyses were accomplished with SAS Statistical Software, SAS Institute, Cary, NC, USA (MANOVA) and Microsoft Excel (Microsoft Corp., paired t-tests.)

3.3 RESULTS

The pairs of excitation-emission wavelengths chosen for this study were 407-592 nm for Uro I, 397-591 nm for Copro III and 410-610 nm for Proto IX. These wavelength pairs provided the most discriminating fluorescence signals for each of the three porphyrins. The percent differences between the actual and measured concentrations of Uro I and Copro III were within 18% for all four trials, however the concentration of Proto IX was consistently under-estimated with the percent differences ranging from 27%

to 60% (Table 3.1).

Table 3.1. Accuracy of the spectrofluorometric method with the wavelength pairs chosen for this study.

	Actual (nM)			Measured (nM)			Percent Difference (%)		
Mixture	Uro I	Copro III	Proto IX	Uro I	Copro III	Proto IX	Uro I	Copro III	Proto IX
1	25	50	75	24	41	55	4	18	27
2	60	20	40	67	22	28	12	10	30
3	40	60	20	41	60	8	3	0	60
4	80	120	40	72	106	20	10	12	50

In the two mixtures containing no Uro I and Copro III, erroneous detection of these two porphyrins occurred. The percent differences between the actual and measured concentrations for Uro I with the wavelength pairs reported by Grandchamp et al. (1980) were within 18% for all four trials, however, the percent differences between the actual and measured concentrations of Copro III and Proto IX ranged from 3% to 110% and 20% to 90%, respectively (Table 3.2).

Table 3.2. Accuracy of the spectrofluorometric method with the wavelengths reported by Grandchamp et al. (1990).

	Actual (nM)			Measured (nM)			Percent Difference (%)		
Mixture	Uro I	Copro III	Proto IX	Uro I	Copro III	Proto IX	Uro I	Copro III	Proto IX
1	25	50	75	25	63	20	0	26	73
2	60	20	40	70	42	4	16	110	90
3	40	60	20	47	69	30	18	15	50
4	80	120	40	80	124	48	0	3	20

The addition of Hepta I and Copro I contributed substantially to the calculated concentrations of Uro I and Copro III with the wavelength pairs chosen for this study (Tables 3.3, 3.4, 3.5).

Table 3.3. Effect of the addition of heptacarboxylate porphyrin I to mixtures of porphyrin standards.

	Actual (nM)				Measured (nM)		
Mixture	Uro I	Copro III	Proto IX	Hepta I	Uro I	Copro III	Proto IX
1	0	83	333	83	154	129	271
2	125	0	292	83	264	50	226
3	104	313	0	83	243	307	0

Table 3.4. Effect of the addition of Coproporphyrin I to mixtures of porphyrin standards.

	Actual (nM)				Measured (nM)		
Mixture	Uro I	Copro III	Proto IX	Copro I	Uro I	Copro III	Proto IX
1	0	83	333	83	1	169	323
2	125	0	292	83	162	78	264
3	104	313	0	83	130	413	0

Table 3.5. Effect of the addition of Heptacarboxylate porphyrin I and Coproporphyrin I to mixtures of porphyrin standards.

	Actual (nM)					Measured (nM)		
Mixture	Uro I	Copro III	Proto IX	Hepta I	Copro I	Uro I	Copro III	Proto IX
1	0	83	333	41	41	71	183	327
2	125	0	292	41	41	214	67	250
3	104	313	0	41	41	217	410	0

A MANOVA model incorporating addition of Hepta I and Copro I as treatment effects (no addition, treatment 1; addition of Hepta I, treatment 2; addition of Copro I, treatment 3) and concentrations of Uro I, Copro III, and Proto IX as dependent variables, was significant for treatment effects (Wilk's Lambda, $F = 7.67$, $p = 0.0056$, $df = 6,8$). Independent contrasts revealed that Hepta I contributed significantly to the calculated concentration of Uro I ($F = 6.16$, $p = 0.048$, $df = 1,2$). The contribution of Copro I to the calculated concentration of Copro III was marginally non-significant ($F = 0.47$, $p = 0.512$, $df = 1,2$). Hepta I had minimal effect on the calculated concentration of Copro III ($p = 0.822$) and, likewise, Copro I had no effect on the calculated concentration of Uro I ($p = 0.726$). Also, addition of Hepta I and Copro I to the porphyrin mixtures had no effect on

the calculated concentrations of Proto IX ($p = 0.765$, and $p = 0.929$, respectively). Qualitative assessment of the spectral scans of all five porphyrins also confirmed these results. Both the excitation and emission spectra of Hepta I and Copro I were nearly identical in position to those of Uro I and Copro III, respectively (Figs. 3.1 and 3.2).

Ten fecal extracts prepared for HPLC analysis were diluted 100 fold and the concentrations of Uro I, Copro III, and Proto IX were calculated with the spectrofluorometric method. For all samples, except for one value for Uro I, the concentrations of the three porphyrins were overestimated significantly by the spectrofluorometric method (Uro I, $p = 0.0011$; Copro III, $p = 0.000002$; Proto IX, $p = 0.0010$) (Table 3.6).

Table 3.6. Comparison of HPLC and spectrofluorometric methods for the determination of porphyrin concentrations. Samples represent fecal extracts of river otters from Prince William Sound, Alaska, USA.

	HPLC Method (μM)			Spectrofluorometric Method (μM)		
Sample	Uro I	Copro III	Proto IX	Uro I	Copro III	Proto IX
C004	0	0.40	0	167	1144	811
C108	0.13	0.72	0	359	981	85
C031	0	0	0	148	409	168
C011	0.46	0.29	0	144	830	408
C063	0.12	2.05	0	63	1153	738
HB23	0.97	2.12	0	0	1100	1100
KI12	0	0.82	0	78	1132	944
HB04	0.41	1.02	4.44	193	937	874
HB01	0.18	0.32	1.26	267	647	213
DI02	0	0	0	126	595	265

Fecal sample extracts prepared for HPLC analysis (500μL) were diluted 100 fold prior to spectrofluorometric analysis. The detection limit for the HPLC method was approximately 10 pmol/500μL (0.020μM).

Values for Proto IX were calculated in eight samples in which Proto IX was not detected with the HPLC method. Values for Uro I and Copro III also were calculated for samples in which these porphyrins were not detected with the HPLC method. Ratios for the measured concentrations of porphyrins in the 10 samples with the spectrofluorometric method were different than the ratios of the concentrations determined with the HPLC method.

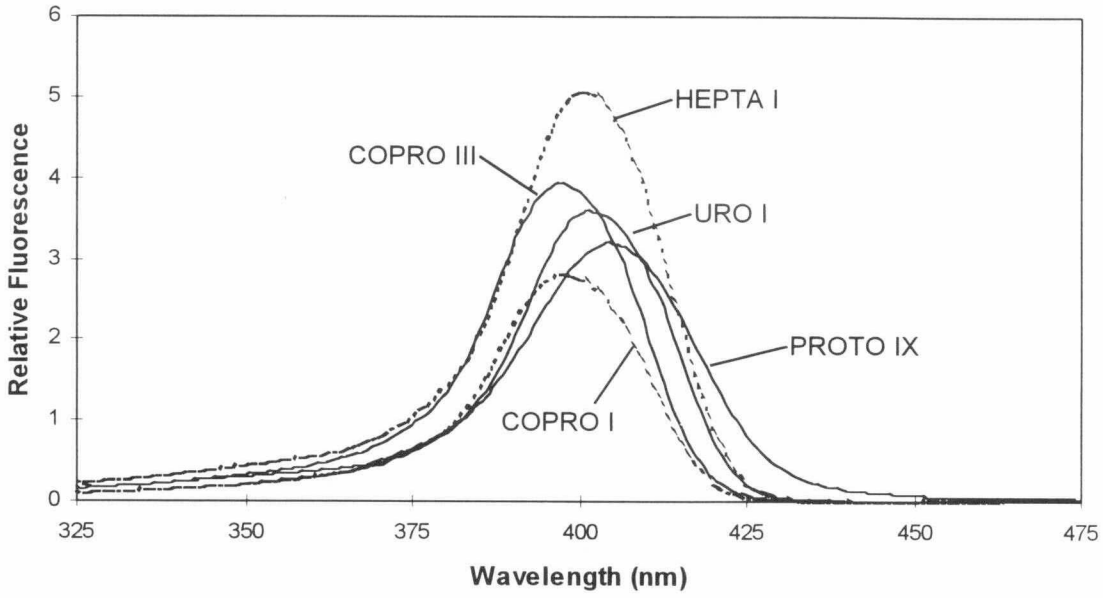

Figure 3.1. Overlay of the excitation spectral scans of Uroporphyrin I, Heptacarboxylate porphyrin I, Coproporphyrin I, Coproporphyrin III and Protoporphyrin IX. The excitation peaks of Heptacarboxylate I and Coproporphyrin I are nearly identical in position to those of Uroporphyrin I and Coproporphyrin III, respectively.

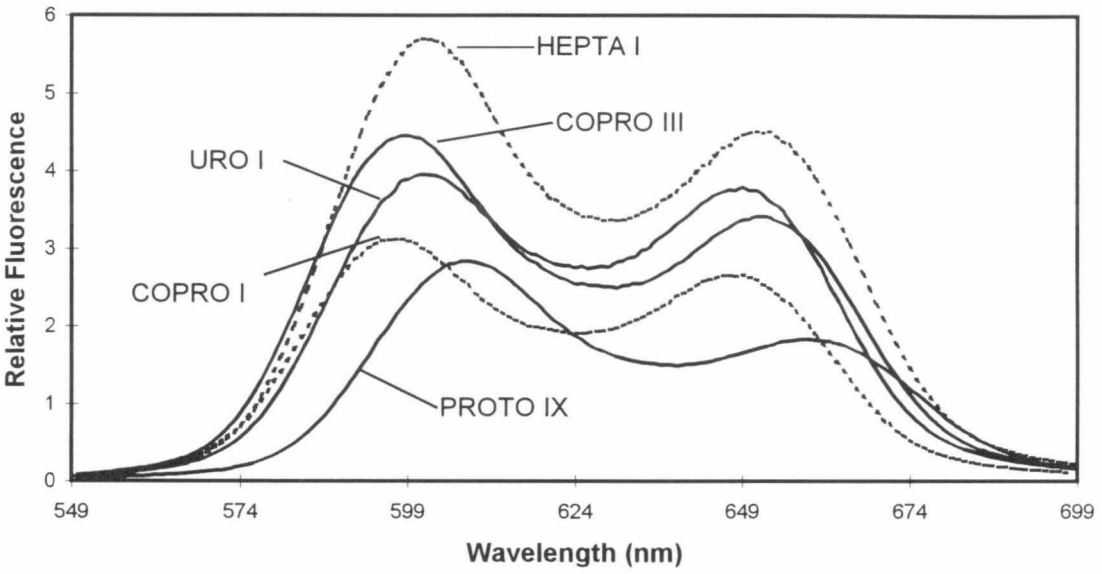

Figure 3.2. Overlay of the emission spectral scans of Uroporphyrin I, Heptacarboxylate porphyrin I, Coproporphyrin I, Coproporphyrin III and Protoporphyrin IX. The emission peaks of Heptacarboxylate I and Coproporphyrin I are nearly identical in position to those of Uroporphyrin I and Coproporphyrin III, respectively.

The contribution of fluorescing matrix constituents in the fecal extracts to the measured concentrations of the porphyrins was qualitatively assessed by comparing spectral scans of whole fecal extracts with chromatographic results from HPLC. The spectrofluorometric emission scan of a whole fecal extract revealed a broad fluorescence signal that merged with the porphyrin peak (Fig. 3.3). All of the HPLC chromatograms for the fecal samples analyzed for this study contained material that eluted prior to the porphyrins (Fig. 3.4). This component of the eluate was fraction collected during HPLC analysis, dried under a stream of air and reconstituted in 1.0 N HCl for spectrofluorometric analysis. The spectrofluorometric emission scan of the fractionated component revealed a fluorescence signal similar to the fluorescence signal observed in the whole fecal extract emission scan (Fig. 3.5).

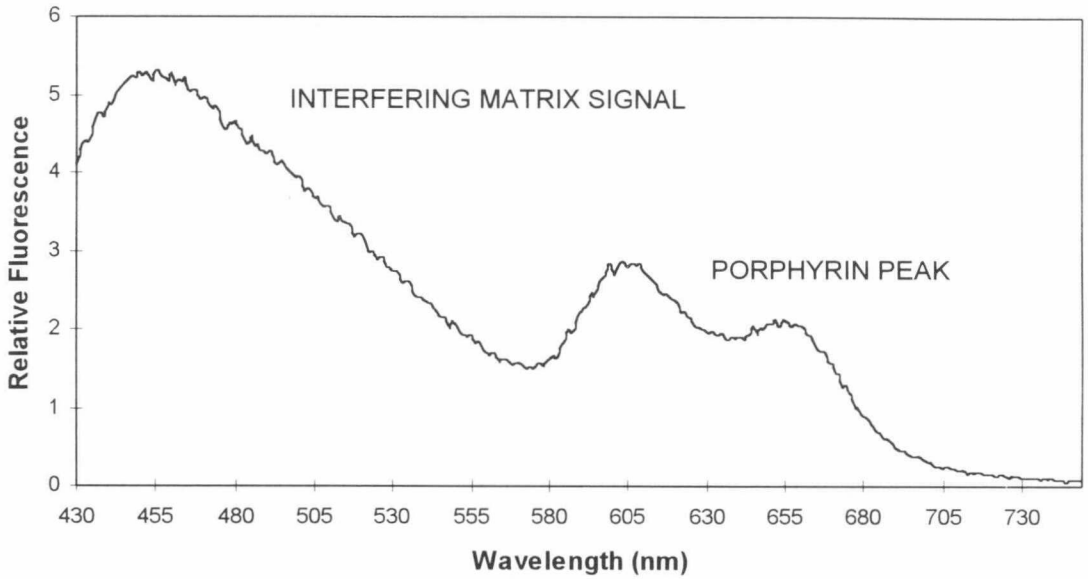

Figure 3.3. Emission spectral scan of a whole extract from a river otter fecal sample from Prince William Sound, Alaska, USA. This scan shows a broad matrix peak merging with the porphyrin peak.

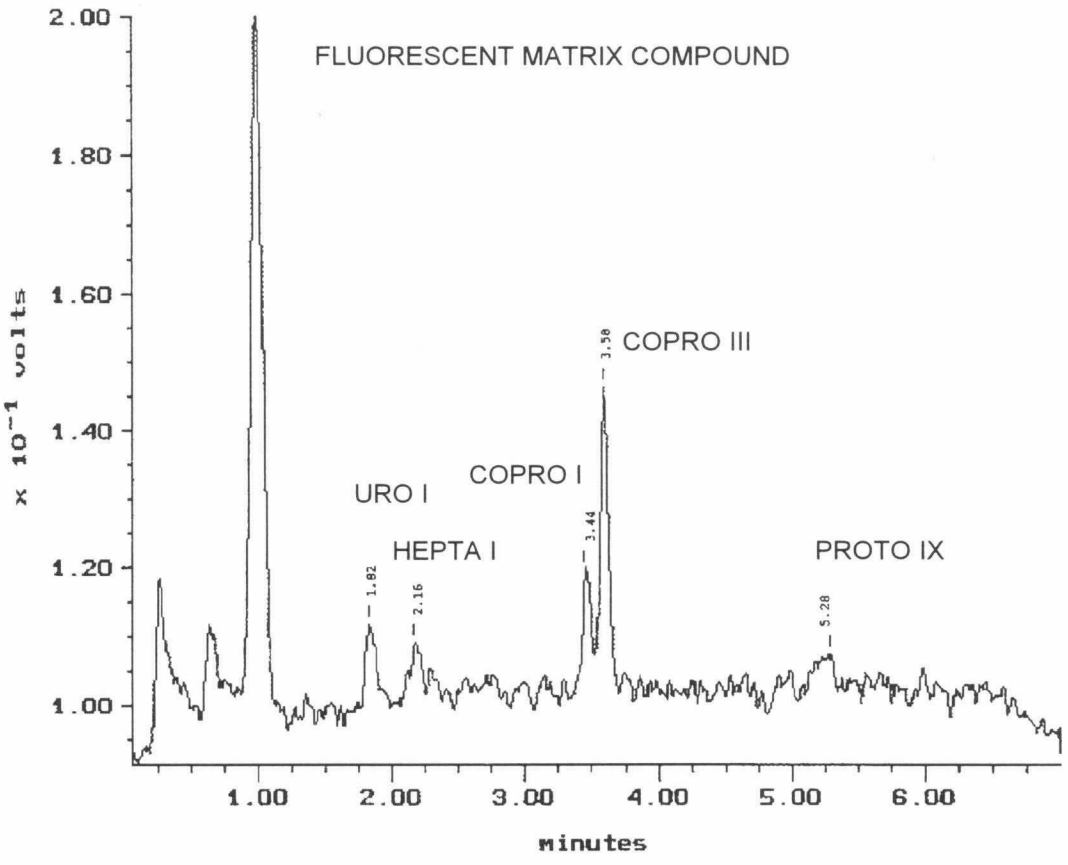

Figure 3.4. HPLC porphyrin profile of a river otter fecal sample from Prince William Sound, Alaska, USA. This chromatogram demonstrates the presence of Hepatacarboxylate porphyrin I and Coproporphyrin I as well as Uroporphyrin I, Coproporphyrin III and Protoporphyrin IX in the fecal extract.

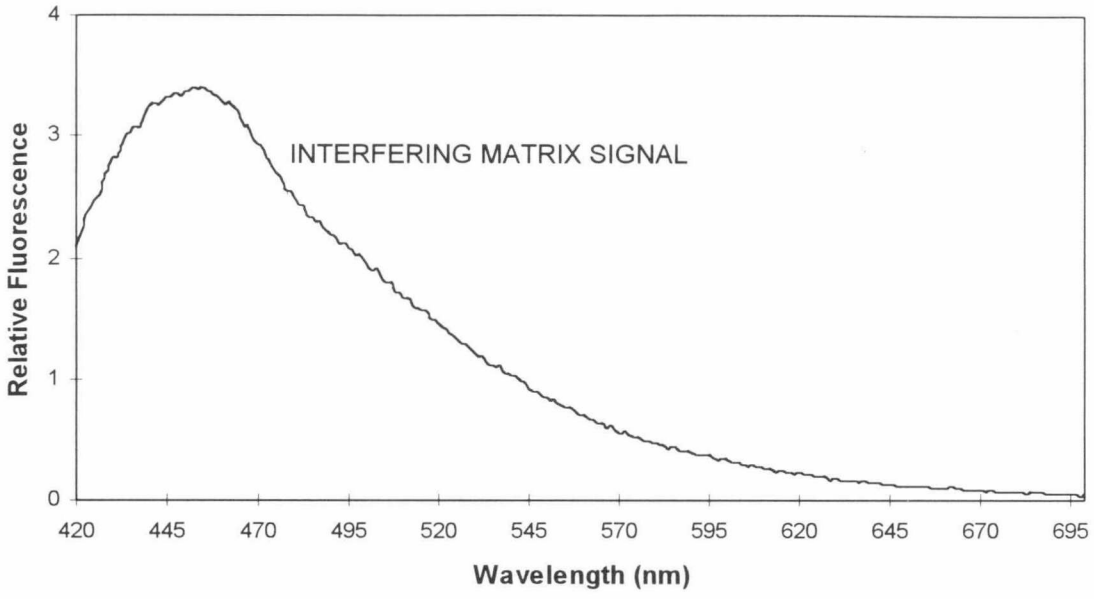

Figure 3.5. Emission scan of an HPLC fraction isolated as a fluorescent peak evident on the HPLC chromatogram. The broad signal produced by this fraction corresponds to a similar signal evident in the emission scans of whole fecal extracts.

3.4 DISCUSSION

The use of the spectrofluorometric method with the pairs of excitation-emission wavelengths chosen for this study resulted in relatively high accuracy for the determination of Uro I and Copro III in mixtures of porphyrin standards at concentrations ranging from 20 to 120 nM; differences between the actual and measured concentrations were within 18%. Consistently lower accuracy was observed for Proto IX at concentrations ranging from 8 to 55 nM with differences between actual and measured concentrations ranging from 27% to 60%. Although, use of the wavelength pairs chosen for this study resulted in higher accuracy on average than with the wavelength pairs reported by Grandchamp et al. (1980), the low accuracy associated with the determination of the concentration of Proto IX precludes the use of the method for sensitive evaluation of the concentration of Proto IX relative to the concentrations of Uro I and Copro III. Furthermore, because of the lower accuracy associated with Proto IX, estimation of total porphyrins (Uro I, Copro III, and Proto IX combined) also would be suspect.

HPLC analysis showed that Hepta I and Copro I as well as Uro I, Copro III, and Proto IX were critical in defining the porphyrin profile in fecal samples of river otters. The results of this study demonstrate that the presence of Hepta I and Copro I contribute significantly to the calculated concentrations of Uro I and Copro III, respectively, using the spectrofluorometric method. Furthermore, because the emission spectra of Hepta I and Copro I are nearly identical in position to the emission spectra of Uro I and Copro III, respectively, this method lacks the sensitivity necessary for more diagnostic studies in which determination of the individual concentrations of Uro I, Hepta I, Copro I and Copro III may be critical in identifying changes in the porphyrin profile. For example, heptacarboxylate porphyrin contributed significantly to the porphyrin profiles in embryo hepatocytes of chickens after exposure to PCBs (Lorenzen et al., 1997). Also, in instances when uroporphyrinogen synthetase is deficient or inhibited, the type I isomer of uroporphyrin may be produced, which, in turn, may result in the accumulation of Copro I

(DeMatteis and Lim, 1994).

The spectrofluorometric assay was not appropriate for the determination of the ratios of porphyrins in the fecal samples of river otters. Fluorescing components of the fecal matrix resulted in elevated and erroneous values for the concentrations of Uro I, Copro III, and Proto IX. Qualitative analysis of the emission spectra of whole fecal extracts showed a fluorescence signal extending from 430 nm to 600 nm, which merged with the porphyrin peak, and most likely contributed to erroneous values. That peak corresponded to the emission spectrum of a matrix compound evident as a peak on the HPLC chromatogram that was fraction collected and analyzed on the spectrofluorometer. This compound could be partially removed by addition of a dilute methanol wash through the C-18 Sep Pak® cartridges to the solid phase extraction-isolation procedure (results not shown). Although the development of more extensive extraction procedures may facilitate the separation of porphyrins from other matrix components, this would preclude the use of the spectrofluorometric assay for rapid and cost efficient analysis of porphyrins.

The solid phase extraction-isolation and HPLC methods described in this study are an alternative to the spectrofluorometric assay, and are relatively rapid and sensitive for quantifying the porphyrins that typically define the porphyrin profile of biological samples. The solid phase extraction-isolation step was developed primarily for sample concentration. Attempts to separate porphyrins from all of the matrix components with the C-18 Sep-Pak cartridges were problematical because the same solvent systems necessary for eluting all of the porphyrins from the cartridges also were those that facilitated the removal of the fecal matrix components. The fluorescing matrix components present in the final eluate, however, did not interfere with HPLC identification and quantification of porphyrins.

The HPLC conditions described in this study incorporating the use of an inexpensive 3-cm long column and short (6 min) run time with 5 min column re-equilibration facilitates rapid separation and identification of porphyrins. The type I and type III isomers of coproporphyrin were discriminated readily by this method. This HPLC

method may serve as a rapid alternative to the spectrofluorometric assay for samples from wildlife containing significant interfering, fluorescing constituents and porphyrins other than Uro I, Copro III, and Proto IX, which contribute to the porphyrin profile.

PROFILES OF FECAL PORPHYRINS IN RIVER OTTERS FOLLOWING THE *EXXON VALDEZ* OIL SPILL

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ABSTRACT

Porphyrin profiles were characterized in fecal samples of river otters (*Lutra canadensis*) inhabiting oiled and nonoiled sites in Prince William Sound, Alaska, USA, during 1990 and 1996. The median level of Coproporphyrin III (Copro III) in fecal samples collected from an oiled area during 1990 was significantly higher than in samples collected from the same oiled area during 1996 and a reference area during 1996 (p = 0.001 and p = 0.010, respectively; one-way analysis of variance). The median level of Copro III in fecal samples collected from the oiled area during 1990 was also higher than in fecal samples collected from a reference area during 1990, however, that difference was not significant (p = 0.096). The decrease in Copro III levels between 1990 and 1996 at the oiled area exhibits a temporal pattern of physiological response similar to that described by other biomarkers measured in river otters at the same oiled site from 1990 through 1992. An overall test of significance that combined probabilities from the analyses of this porphyrin study with those from the other biomarker studies revealed a significant difference in response between oiled and nonoiled areas for 1990 ($p < 0.01$). Results of this study demonstrate that, as part of a suite of biomarkers, changes in the profile of fecal porphyrins may serve as effective indicators of physiological response that may contribute to the development of a health assessment of wild populations of river otters.

Keywords--Porphyrin profiles Biomarker *Lutra canadensis* Prince William Sound, Alaska *Exxon Valdez*

4.1 INTRODUCTION

Research following the Exxon Valdez oil spill in March 1989 has assessed biochemical and physiological effects within organisms resulting from chronic exposure to persistent levels of oil in the coastal marine environment. River otters (*Lutra canadensis*) serve as an excellent sentinel species for biomarker studies designed to assess the effects of oil exposure in Prince William Sound because these mustelids occupy a high trophic position within the marine ecosystem and feed on intertidal and subtidal organisms (Ben-David et al., 1998; Bowyer et al., 1994). Furthermore, river otters are ubiquitous within Prince William Sound as well as the Gulf of Alaska (Bowyer et al., 1995), and therefore sampling protocols at both oiled and nonoiled sites may be readily developed. River otters serve as effective indicators of the presence of oil in the coastal environment, its bioavailability, and its potential effects on other coastal vertebrates.

Several studies of biomarkers have demonstrated biochemical and physiological responses consistent with the effects of contaminant exposure in river otters inhabiting oiled sites within Prince William Sound. Levels of blood haptoglobins, interleuken-6 ir, and a series of blood enzymes were elevated in river otters inhabiting oiled sites compared with nonoiled sites in 1991 (Duffy et al., 1994 a). River otters from oiled sites also had lower body mass compared with nonoiled sites in 1991 (Duffy et al., 1993). Total porphyrins in whole fecal extracts from river otters were elevated in samples collected from oiled sites compared with nonoiled sites during 1990 (Blajeski et al., 1996).

The potential for certain chemical exposures to result in an alteration of the heme biosynthetic pathway leading to the excretion of porphyrin precursors, particularly in humans and laboratory animals, has been well established (Marks, 1985; Silbergeld and Fowler, 1987). Several studies have focused on the use of patterns of porphyrin excretion as a biomarker of contaminant exposure in wildlife. In a controlled field study, methylmercury administration resulted in significantly elevated levels of renal and hepatic porphyrins in European starlings (*Sturnus vulgaris*) (Akins et al., 1993). Mediterranean

crabs (*Carcinus aestuarii*) collected in the wild and studied in a laboratory setting had decreased levels of fecal porphyrins following exposure to benzo(a)pyrene, polychlorobiphenyls, and methylmercury (Fossi et al., 1997). Highly carboxylated porphyrins were elevated in herring gull chicks (*Larus argentatus*) from a site contaminated with polyhalogenated aromatic hydrocarbons compared with a noncontaminated site within the Great Lakes (Kennedy and Fox, 1990).

This study was designed to evaluate the effects of crude oil exposure on the pattern of fecal porphyrin excretion in river otters in a field setting through the use of refined analytical techniques that facilitate the separation, identification, and quantification of individual porphyrins with high-performance liquid chromatography (HPLC). We hypothesized that the elevated levels of total porphyrins observed in river otters inhabiting oiled sites compared with nonoiled sites within Prince William Sound, Alaska, following the *Exxon Valdez* oil spill (Blajeski et al., 1996) could be attributed to an increase in the excretion of individual porphyrins, which may be caused by specific alterations of the heme biosynthetic pathway. We characterized the porphyrin profiles in fecal samples of river otters and quantified levels of individual porphyrins to determine if differences in patterns of fecal porphyrin excretion existed between river otters inhabiting oiled and nonoiled sites. We also hypothesized that as the availability of oil decreased over time, the patterns of porphyrin excretion would become similar in fecal samples from river otters inhabiting oiled and nonoiled sites. To determine if recovery was occurring over time, we compared patterns of porphyrin excretion in fecal samples of river otters collected from the same oiled area during 1990 and 1996.

4.2 METHODS

Sample Collection and Preparation

Fecal samples were taken from the frozen archive at the Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, Alaska, USA. The fecal samples were

collected from the same oiled site on northern Knight Island (60°30'N, 147°40'W) during 1990 and 1996, from a nonoiled reference site in Esther Passage (60°53N, 147°55'W) during 1990, and from a second reference site in Jackpot Bay (60°25N, 147°30'W) during 1996. Ninety six samples were collected from specific locations called latrine sites where river otters socialize and deposit feces and anal secretions (Ben-David et al., 1998). The fecal samples were placed in plastic bags and stored in a freezer at -70°C. The fecal samples were lyophilized for 24 hours prior to porphyrin extraction and HPLC analysis.

Reagents

ACS-grade hydrochloric acid, sodium phosphate, acetonitrile and acetone were used for porphyrin extraction from the fecal samples. ACS-grade ammonium acetate, and HPLC-grade methanol were used for HPLC analysis. Milli Q-18 water was used for all solvent preparations.

Standards

The porphyrin standards used for determination and quantification of fecal porphyrins included a standard chromatographic marker kit containing the number I isomers of 8, 7, 6, 5, 4 carboxylate porphyrins and mesoporphyrin IX, as well as coproporphyrin III, deuteroporphyrin IX and protoporphyrin IX. All porphyrin standards were purchased from Porphyrin Products, Logan, Utah.

Equipment

Separation of porphyrins was facilitated by an HPLC system consisting of two Waters 510 pumps (Waters Corp., Milford, MA, USA) a Rheodyne 7125 injector valve equipped with a 5 uL injection loop (Rheodyne Corp., Cotati, CA, USA) and a 3 cm by 0.46 cm Luna C-18 column equipped with a Security Guard cartridge system (Phenomenex Corp., Torrance, CA, USA). Porphyrins were detected with a McPherson FL-748 fluorescence detector equipped with a 405 nm excitation cut-off filter, a 620 nm

interference emission filter with a 10 nm bandwidth and a red sensitive photomultiplier tube (McPherson Corp., Chelmsford, MA, USA). Baseline 810 and Maxima 820 chromatographic software (Waters Corp.) were used for system control and peak integration. Sample concentration was facilitated with 500 mg trifunctional C-18 (tC-18) solid-phase extraction columns (Sep Paks®; Waters Corp.) attached to a Supelco Visiprep vacuum manifold (Supelco Corp., Bellefonte, PA, USA).

Porphyrin Analysis

Porphyrin extraction and isolation from the fecal samples was accomplished with a modification of the procedure described by Bowers et al. (1992). Porphyrins were extracted with HCl, concentrated on the Sep Pak® cartridges and eluted into cryogenic tubes with a polar solvent that was evaporated to produce a dry residue containing the porphyrins. Five milliliters of 6 N HCl were added to approximately 1.0 g of dry fecal material and the sample was macerated with a glass rod for approximately 1 min. The sample was mixed with a bench top vortex for 1 min, sonicated in a water bath for 5 min, and mixed again for 1 min. Five milliliters of sodium phosphate buffer (0.01 M, pH 3.5) was added to the sample. The sample was mixed again for 1 min and centrifuged for 10 min at 4,000 rpm. The coarse pellet was removed from the centrifuge tube and the sample was centrifuged again for 10 min at 4,000 rpm.

Sep Pak® cartridges were prepared by washing first with 7 ml of acetonitrile followed by 7 ml of sodium phosphate buffer (0.01 M, pH 3.5). Eight milliters of the fecal supernatant was delivered to the Sep Paks® and allowed to gravity feed. The Sep Paks were washed with 3 ml of sodium phosphate buffer (0.01 m, pH 3.5) to facilitate complete delivery of the supernatant to the Sep Pak®, followed by 7 ml of sodium phosphate buffer (0.01M, pH 7.5) to enhance recovery of the porphyrins. The concentrate containing the porphyrins at the top of the Sep Paks® was eluted into 5 ml cryogenic centrifuge tubes with 1 ml of acetonitrile, followed by 0.5 ml of acetonitrile : 1.0 N HCl (1:1, v/v), and finally 1 ml of acetone under vacuum. The tubes containing the eluates were placed in a

water bath at 55°C and evaporated to a dry residue under a stream of air. The dry residues containing the porphyrins were stored frozen at -70°C. For HPLC analysis, the dry residues were reconstituted in 500 μL of 6 N HCl and injected directly into the HPLC system.

HPLC Analysis

The HPLC method used was a modification of the procedure described by Kennedy and James (1993). Separation of porphyrins was facilitated with a 6-min gradient elution and a two-component mobile phase consisting of ammonium acetate (1.0 M, pH 5.16) as solvent A and 100% methanol as solvent B. Gradient elution commenced upon injection and proceeded from 25% B at time zero to 50% at 1.0 min, then to 95% B at 4.0 min, remained at 95% B for 1.5 min and returned to 25% B at 6 min. The column was allowed to re-equilibrate for 5 min at 25% B before the next injection. The concentration of porphyrins in each fecal sample was calculated with a seven-point calibration curve (0.0 μM to 3.0 μM) developed with solutions of porphyrin standards dissolved in 6 N HCl.

Statistical Analysis

A one-tailed analysis of variance (ANOVA; PROC GLM/CONTRASTS, SAS, SAS Institute, Cary, NC, USA) was used to evaluate Copro III concentrations between oiled and nonoiled locations and between sampling years. Data on Copro III was ranked (Conover and Iman, 1981) prior to performing the ANOVA (PROC RANK, SAS). Multivariate analysis of variance (MANOVA; PROC GLM/MANOVA, SAS) testing location, year, and location-year interactions was used to assess the contribution of the number I isomer porphyrins to the fecal profile between locations and years. Probabilities of ≤ 0.05 were considered significant for the ANOVA and MANOVA models. To test for overall significance in biomarker response described by Copro III levels as well as levels of haptoglobins and body mass measurements reported in previous studies between the oiled

and nonoiled areas during 1990, a combined test of probabilities was performed as described by Sokal and Rohlf (1980).

4.3 RESULTS

The porphyrins detected in the fecal samples from river otters were primarily Uroporphyrin I (Uro I), Heptacarboxylate porphyrin I (Hepta I), Coproporphyrin I (Copro I), Coproporphyrin III (Copro III) and Protoporphyrin IX (Proto IX) (Fig. 4.1). Proto IX was detected in 30 % of the 96 samples and Deuteroporphyrin was detected in 4 % of the 96 samples. The mean levels of porphyrins detected ranged from 0.03 nmol/g for Uro I and Hepta I in the Knight Island 1996 oiled area to 1.13 nmol/g for Copro III in Knight Island 1990 oiled area (Table 4.1).

Table 4.1. Mean concentration of porphyrins detected in fecal samples of river otters from Prince William Sound, Alaska, USA.

	Concentration (nmol/g; Mean ± Standard Deviation)			
Porphyrin	Knight Island 1990 (n = 22)	Knight Island 1996 (n = 28)	Esther Passage 1990 (n = 23)	Jackpot Bay 1996 (n = 23)
Uro I	0.14 ± 0.12	0.03 ± 0.05	0.09 ± 0.09	0.18 ± 0.18
Hepta I	0.20 ± 0.27	0.03 ± 0.07	0.08 ± 0.17	0.06 ± 0.07
Copro I	0.23 ± 0.27	0.37 ± 0.75	0.21 ± 0.28	0.09 ± 0.13
Copro III	1.13 ± 0.94	0.43 ± 0.41	0.93 ± 0.98	0.54 ± 0.55

The median value for Copro III was highest at Knight Island 1990 (an oiled area) (Fig. 4.2). The overall ANOVA model was significant for the location-year effect (one-tailed; $F = 3.57$; d.f. = 3, 92; $p = 0.008$) indicating that Copro III levels were significantly different among locations and years. Individual one-tailed contrasts for year and location effects revealed Copro III levels at Knight Island in 1990 were significantly higher than those at Knight Island in 1996 ($p = 0.001$), and the Jackpot Bay reference area ($p = 0.010$). Although the median level of Copro III was higher at Knight Island in 1990 than the median level of Copro III at Esther Passage in 1990, the difference was not significant (p

= 0.096). There were no significant differences between areas or years for Uro I, Hepta I or Copro I (MANOVA; location effect, Wilks' Lambda $p = 0.0637$; year effect, Wilks' Lambda $p = 0.0516$). Furthermore, the MANOVA model revealed a location-year interaction effect (Wilks' Lambda p = 0.0007) indicating a reversal in trend for the concentration of these porphyrins between the oiled and nonoiled areas between 1990 and 1996. A test to evaluate differences in biomarker response that combined probabilities of separate tests of significance for differences in Copro III levels, haptoglobin levels, and body mass revealed an overall significant difference in biomarker response between oiled and nonoiled areas during 1990 ($X^2 = 17.5623$, df = 6, $p < 0.01$).

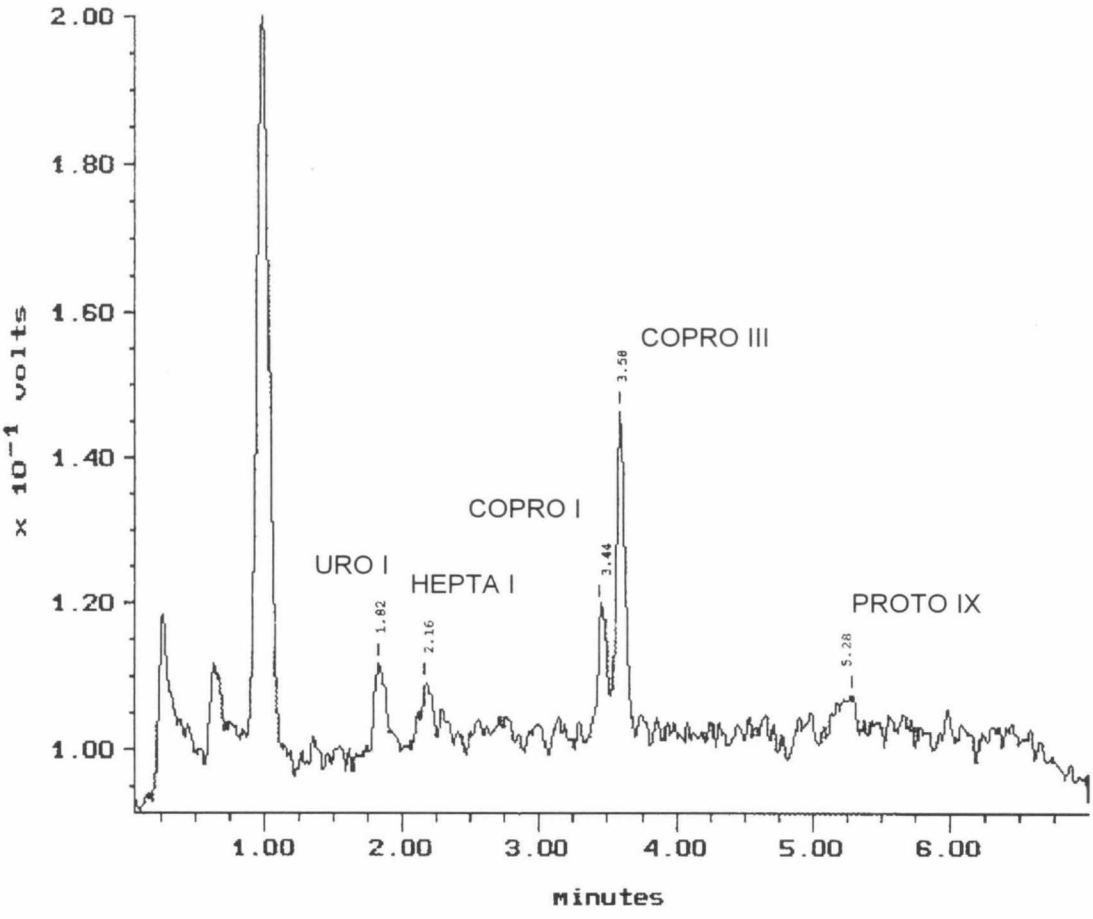

Figure 4.1. Chromatogram of the porphyrin profile in a fecal sample from a river otter collected in the oiled area (Knight Island) of Prince William Sound, Alaska, USA, during 1990. Both Coproporphyrin I and Coproporphyrin III as well as Uroporphyrin I, Heptacarboxylate porphyrin I and Protoporphyrin IX are discriminated.

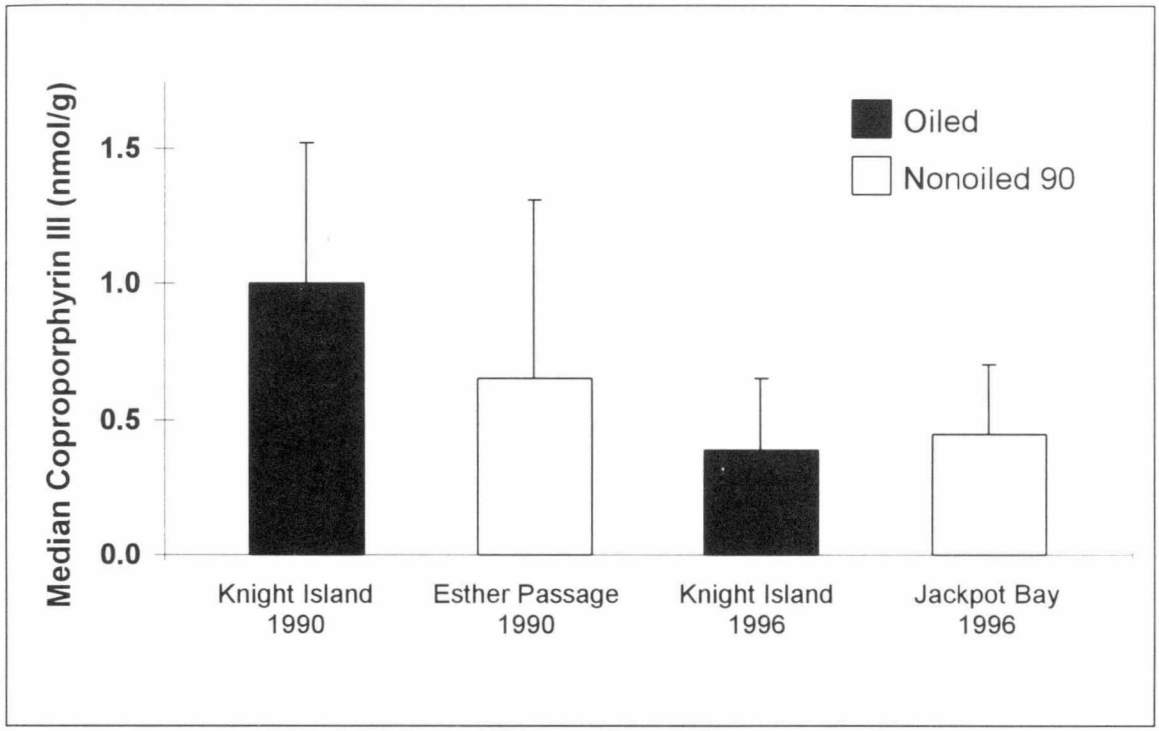

Figure 4.2. Median concentrations of Coproporphyrin III for each year and location. Error bars represent one-half the interquartile distance.

4.4 DISCUSSION

In a previous study, river otters in an oiled area of Prince William Sound had higher levels of total porphyrins in fecal samples than river otters in a nonoiled area during 1990, and qualitative HPLC analysis indicated that elevated coproporphyrin excretion was most prevalent in the fecal samples of river otters inhabiting the oiled area (Blajeski et al., 1996). Moreover, low but significantly different concentrations of oil-related hydrocarbons occurred on the pelage of river otters inhabiting oiled relative to nonoiled areas of Prince William Sound, suggesting that chronic exposure to oil was occurring 8 years following the spill (Duffy et al., 1999).

The refined extraction and HPLC techniques described herein allowed discrimination between the number I and III isomers of coproporphyrin (Fig. 4.1) and facilitated the determination that, specifically, Copro III may have been responsible for the higher total porphyrin levels observed previously (Table 4.1). Levels of Copro III were significantly higher in river otters from Knight Island in 1990 than that same area in 1996, and at Jackpot Bay in 1996 (reference area). Copro III levels also were higher in river otters at Knight Island in 1990 than at Esther Passage in 1990, however, that difference was not significant.

Levels of Copro III in fecal samples collected from the oiled area during 1990 and 1996 followed a similar temporal pattern of physiological response as that observed with other biomarkers measured in river otters at the same oiled area from 1990 through 1992. River otters inhabiting the oiled area during 1990 had higher levels of haptoglobin, an acute phase protein indicative of tissue damage, and lower body mass than river otters inhabiting the nonoiled area (Duffy et al., 1993; 1994a). A follow-up study, however, reported no significant differences in haptoglobin levels and body mass of river otters between the same oiled and nonoiled sites by 1992 (Duffy et al., 1994b).

These same studies also reported higher haptoglobin levels and lower body mass in river otters inhabiting the Esther Passage reference area during 1990 than during 1992.

The observation of higher levels of haptoglobins and lower body mass in river otters at Esther Passage during 1990 corresponds to the observation of Copro III levels in fecal samples collected from Esther passage during 1990 similar to Copro III levels in fecal samples collected from the oiled area during 1990. With a test of combined probabilities (Sokal and Rohlf, 1980), however, an overall significant difference in biomarker response (Copro III, haptoglobins, body mass) occurred between the Knight Island oiled area and the Esther Passage nonoiled area during 1990.

Results of previous biomarker studies suggest that Esther Passage may not have been an appropriate reference area for assessing oil related effects in Prince William Sound following the *Exxon Valdez* oil spill (Duffy et al., 1994b). An image taken by a LANDSAT Thematic Mapper demonstrated the presence of oil at the southern terminus of Esther passage 2 weeks after the *Exxon Valdez* oil spill (Stringer et al., 1992). River otters sampled in Esther Passage may have been exposed to this oil.

The results of this study correspond to the results other biomarker studies of river otters inhabiting both oiled and nonoiled study areas of Prince William Sound following the *Exxon Valdez* oil spill and provide evidence that levels of fecal porphyrins may serve as an effective biomarker of physiological stress that may contribute to a health assessment of wild river otters. Further studies will be necessary to establish a dose-response relationship between oil exposure and changes in the profile of fecal porphyrins in wild river otters.

PROFILES OF FECAL PORPHYRINS IN RIVER OTTERS OF NORTHERN LYNN CANAL, SOUTHEAST ALASKA

ABSTRACT

Profiles of porphyrins were characterized in fecal samples of river otters inhabiting shorelines of northern Lynn Canal in southeast Alaska. Porphyrins detected in the fecal samples included Uroporphyrin I (Uro I), Heptacarboxylate porphyrin I (Hepta I), Hexacarboxylate porphyrin I (Hexa I), Pentacarboxylate porphyrin I (Penta I), Coproporphyrin I (Copro I), Coproporphyrin III (Copro III), and Protoporphyrin IX (Proto IX). Mean detected levels of porphyrins ranged from 0.02 nmol/g for Penta I to 1.74 nmol/g for Proto IX. Profile characteristics of the eight through four carboxylate porphyrins in the river otter fecal samples collected in Lynn Canal were similar to the porphyrin profiles characterized in fecal samples collected at a reference area in Prince William Sound, Alaska. A higher proportion of the fecal samples collected in Lynn Canal, however, had detectable levels of Proto IX than the fecal samples collected at the reference area in Prince William Sound. The results of this study indicate first, that a complete porphyrin profile may be characterized in most fecal samples collected fresh in the field and second, that the age and potential deterioration of fecal samples may be of consideration for efficient recovery of Proto IX. The results of this study also contribute to the establishment of a baseline for endogenous porphyrin excretion in fecal samples of river otters, which may be applied to the evaluation of changes in porphyrin excretion that may result from the effects of crude oil exposure in Prince William Sound.

5.1 INTRODUCTION

To develop the use of the profile of fecal porphyrins of river otters (*Lutra canadensis*) as a biomarker of the effects of crude oil exposure in Prince William Sound, Alaska, USA, following the *Exxon Valdez* oil spill, a reliable baseline documenting endogenous excretion of fecal porphyrins was necessary. Two areas in Prince William Sound, Esther Passage and Jackpot Bay, which were not effected by *Exxon Valdez* oil, were chosen as reference areas for biomarker research in river otters. Porphyrin levels at Esther Passage during 1990, 1 year following the oil spill, were higher than porphyrin levels at Jackpot Bay during 1996. Also, Copro III levels at Esther Passage, although lower, were not significantly different from Copro III levels at Knight Island, a heavily oiled area, during 1990. The high levels of porphyrins at Esther Passage in 1990 also corresponded to high haptoglobin values reported in another biomarker study conducted during 1990, which suggested that Esther Passage was not a reliable reference area (Duffy et al., 1994b). The discrepancy in porphyrin levels between the Esther Passage and Jackpot Bay reference areas as well as the observation of correspondingly high levels of haptoglobins during 1990 in Esther Passage necessitated the investigation of a reference area that would provide a reliable baseline for fecal porphyrin excretion. Lynn Canal in northern southeast Alaska (Fig. 5.1) was determined to be a suitable reference area for sampling river otters because the coastal habitat is similar to that in Prince William Sound and Lynn Canal had not received a major contaminant event. The purpose of this study was to characterize the profile of fecal porphyrins in river otters inhabiting northern Lynn Canal to help establish a baseline profile that may be applied to the evaluation of fecal porphyrin excretion in river otters inhabiting oiled and nonoiled areas of Prince William Sound.

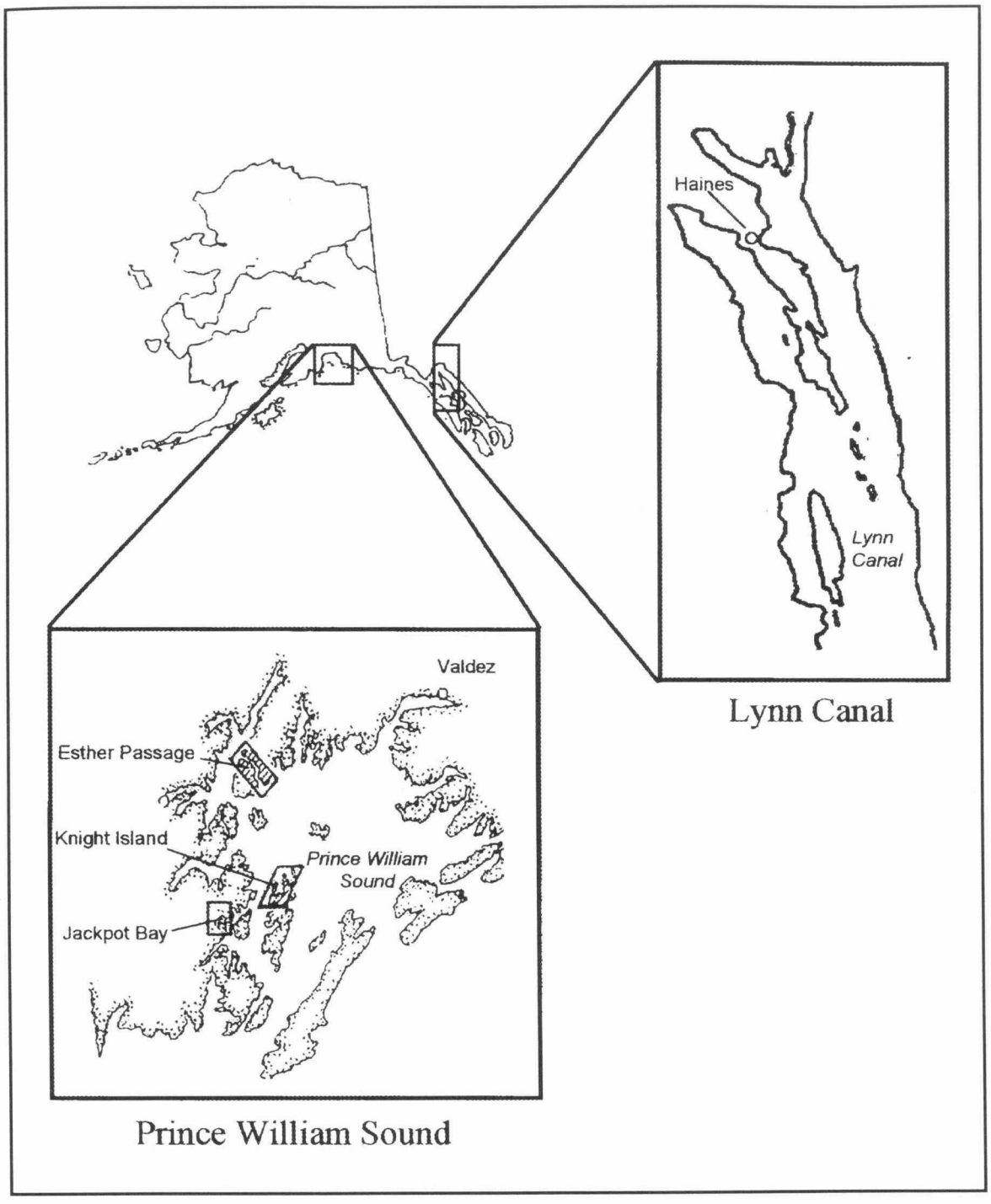

Figure 5.1. Field locations for fecal porphyrin studies in Prince William Sound and Lynn Canal, Alaska, USA.

5.2 METHODS

Sample Collection and Preparation

Fecal samples from river otters were collected from shorelines extending from Lutak Inlet to Sullivan Island in northern Lynn Canal during June and July of 1998. Fecal samples were placed in plastic bags and stored frozen at -70°C until analysis. Prior to HPLC analysis, the fecal samples were lyophilized for 24 hours.

Reagents and standards

ACS-grade hydrochloric acid, sodium phosphate, acetonitrile and acetone were used for porphyrin extraction from the fecal samples. ACS-grade ammonium acetate, and HPLC-grade methanol were used for HPLC analysis. Milli Q-18 water was used for all solvent preparations.

The porphyrin standards used for determination and quantification of fecal porphyrins included a standard chromatographic marker kit containing the number I isomers of 8, 7, 6, 5, 4 carboxylate porphyrins and mesoporphyrin IX, as well as coproporphyrin III, deuteroporphyrin IX and protoporphyrin IX. All porphyrin standards were purchased from Porphyrin Products, Logan, Utah, USA.

Equipment

Separation of porphyrins was facilitated by an HPLC system consisting of two Waters 510 pumps (Waters Corp., Milford, MA, USA), a Rheodyne 7125 injector valve equipped with a 5 uL injection loop (Rheodyne Corp., Cotati, CA, USA) and a 3 by 0.46-cm Luna C-18 column equipped with a Security Guard cartridge system (Phenomenex Corp., Torrance, CA, USA). Porphyrins were detected with a McPherson FL-748 fluorescence detector equipped with a 405 nm excitation cut-off filter, a 620 nm interference emission filter with a 10 nm bandwidth and a red sensitive photomultiplier tube (McPherson Corp., Chelmsford, MA, USA). Baseline 810 and Maxima 820

chromatographic software (Waters Corp.) were used for system control and peak integration. Sample concentration was facilitated with 500-mg trifunctional C-18 (tC-18) solid-phase extraction columns (Sep-Paks®; Waters Corp.) attached to a Supelco Visiprep vacuum manifold (Supelco Corp., Bellefonte, PA, USA).

Porphyrin Analysis

Porphyrin extraction and isolation from the fecal samples were accomplished with a modification of the procedure described by Bowers et al. (1992). Porphyrins were extracted with HCl, concentrated on the Sep-Pak® cartridges and eluted into cryogenic tubes with a polar solvent that was evaporated to produce a dry residue containing the porphyrins. Five milliliters of 6 N HCl were added to 1.0 g of dry fecal material and the sample was macerated with a glass rod for approximately 1 min. The sample was mixed with a bench top vortex for 1 min, sonicated in a water bath for 5 min, and mixed again for 1 min. Five milliliters of Milli Q-18 water were added to the sample. The sample was mixed again for 1 min and centrifuged for 10 min at 4,000 rpm. Two milliliters of the supernatant were delivered to a microcentrifuge tube and centrifuged at 7,000 rpm for 10 min.

Sep-Pak® cartridges were prepared by washing first with 7 ml of acetonitrile followed by 7 ml of sodium phosphate buffer (0.01 M, pH 3.5) after which 1.6 ml of the fecal supernatant was allowed to gravity feed. The Sep-Paks® were washed with 3 ml of sodium phosphate buffer (0.01 m, pH 3.5) to facilitate complete delivery of the supernatant to the Sep-Pak®, followed by 7 ml of sodium phosphate buffer (0.01M, pH 7.5) to enhance recovery of the porphyrins. The concentrate containing the porphyrins at the top of the Sep-Paks® was eluted into 5 ml cryogenic centrifuge tubes with 1 ml of acetonitrile, followed by 0.5 ml of acetonitrile : 1.0 N HCl (1:1, v/v), and finally 1 ml of acetone under vacuum. The tubes containing the eluates were placed in a water bath at 55°C and evaporated to a dry residue under a stream of air. The dry residues containing

the porphyrins were stored frozen at -70°C. For HPLC analysis, the dry residues were reconstituted in 100 μL of 6 N HCl and injected directly into the HPLC system.

HPLC Analysis

The HPLC method used was a modification of the procedure described by Kennedy and James (1993). Separation of porphyrins was facilitated with a 6-min gradient elution and a two-component mobile phase consisting of ammonium acetate (1.0 M, pH 5.16) as solvent A and 100% methanol as solvent B. Gradient elution commenced upon injection and proceeded from 25% B at time zero to 50% at 1.0 min, then to 95% B at 4.0 min, remained at 95% B for 1.5 min and returned to 25% B at 6 min. The column was allowed to re-equilibrate for 5 min at 25% B before the next injection. The concentration of porphyrins in each fecal sample was calculated with a seven-point calibration curve (0.0 μM to 3.0 μM) developed with solutions of porphyrin standards dissolved in 6 N HCl.

5.3 RESULTS

The porphyrin profile in the river otter fecal samples collected in Lynn Canal included Uro I, Hepta I, Hexa I, Penta I, Copro I, Copro III and Proto IX. The percentages of samples containing detectable levels of the individual porphyrins ranged from 17% for Penta I to 79% for Proto IX (Table 5.1 a). Mean levels of the porphyrins detected ranged from 0.02 nmol/g for Penta I to 1.74 nmol/g for Proto IX (Table 5.1 b).

Table 5.1. Percentage of fecal samples in which porphyrins were detected (a) and mean levels of detected porphyrins in fecal samples (b) of river otters in Lynn Canal, Southeast Alaska, USA.

	Porphyrin						
	Uro I	Hepta I	Hexa I	Penta I	Copro I	Copro III	Proto IX
a. Detected (%)	37.5	29.2	29.2	16.7	75.0	79.2	79.2
b. Mean (nmol/g*)	0.04	0.05	0.03	0.02	0.54	0.50	1.74
± S. E.	± 0.02	± 0.02	± 0.01	± 0.01	± 0.20	± 0.10	± 0.35

* dry weight

n = 24

5.4 DISCUSSION

The mean levels of porphyrins measured in the river otter fecal samples collected in Lynn Canal were similar to the mean levels of porphyrins measured previously in fecal samples collected at Jackpot Bay, a reference site, and Knight Island, an oiled sited, in Prince William Sound, Alaska, during 1996 (Fig. 5.1). Proto IX was detected in 79% of the fecal samples collected in Lynn Canal. In comparison, Proto IX was detected in only approximately 25% of the samples at Knight Island and 30% of the samples at Jackpot Bay during 1996. Only fresh samples were collected from sites in Lynn Canal and saved for analysis. This could indicate a real difference in the profile of fecal porphyrins between these sites. Alternatively, these results may indicate that deterioration of the fecal samples from the Jackpot Bay and Knight Island areas may have occurred prior to collection in the field or during long term storage or handling. Proto IX is degraded rapidly by bacteria to other dicarboxylic porphyrins (Beukeveld et al., 1987). Because the mean levels of the four through eight carboxylic porphyrins in the fecal samples collected in Lynn Canal were similar to the levels of the same porphyrins in the fecal samples from the Jackpot Bay and Knight Island areas, these results also suggest that degradation or stability of these porphyrins may be of less importance. These results contribute to the establishment of a baseline for porphyrin excretion in fecal samples of coastal marine river otters in Alaska

that may be useful for evaluating changes in the porphyrin profile caused by the effects of contaminant exposure. These results also highlight the importance of selecting an appropriate reference site for comparative studies.

FECAL PORPHYRINS IN RIVER OTTERS EXPERIMENTALLY EXPOSED TO WEATHERED CRUDE OIL

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ABSTRACT

Porphyrin profiles were characterized in fecal samples from river otters (*Lutra canadensis*) experimentally exposed to weathered crude oil to determine effects on heme synthesis. Fifteen male river otters were divided into three groups of five individuals representing a control group, a low-dosage group that received 5 mg/kg body mass of oil per day, and a high-dosage group that received 50 mg/kg body mass of oil per day. Mean levels of Copro III and Protoporphyrin IX (Proto IX) in fecal samples collected from all experimental river otters were higher throughout the experimental period than levels of Copro III and Proto IX in fecal samples collected previously at two field sites. No statistically significant differences in levels of Copro III and Proto IX occurred between treatment groups. Our results suggest that porphyrin excretion in wild river otters may be effected by physiological changes associated with captive conditions. These differences may be due to natural factors such as physiological cycles or nutrition, or anthropogenic stress associated with captivity. The sensitivity of porphyrin profiles in detecting the effects of contaminants such as oil suggests that further research is needed to establish a baseline for porphyrin excretion in river otters that is consistent under field and captive-sampling conditions.

6.1 INTRODUCTION

Numerous studies designed to assess the effects of oil exposure in river otters (*Lutra canadensis*) following the *Exxon Valdez* oil spill in Prince William Sound, Alaska, USA, demonstrated physiological and ecological responses consistent with contaminant exposure in animals inhabiting oiled areas of the sound. River otters inhabiting oiled areas had higher levels of haptoglobins, interleukin 6-ir, other blood enzymes, and fecal porphyrins directly following the oil spill (Duffy et al., 1993; 1994a; Blajeski et al., 1996) as well as changes in diet composition and use of latrine sites (Bowyer et al., 1994; 1995). Although those studies provided a wealth of information that contributed to a health assessment of river otters inhabiting oiled areas of Prince William Sound, as field studies, they could not establish a dose-response relationship, which is central in a toxicological model linking specific contaminants with observed effects (Hugget et al., 1992; Fossi and Leonzio, 1994, Rand, 1995). Thus, a controlled dosing experiment was initiated to evaluate the effects of Alaskan North Slope weathered crude oil administered via the diet of river otters. The conditions of the experiment were designed to simulate conditions of chronic exposure to crude oil persistent in oiled areas of Prince William Sound. The analysis of a suite of biomarker measurements and behavioral observations was proposed to provide an assessment of the effects of weathered oil on the health of river otters. This report evaluates the use of porphyrin profiles as a biomarker of the effects of oil exposure.

Heme biosynthesis proceeds through a series of tetrapyrollic pigments called porphyrinogens, which are subsequently oxidized to porphyrins when produced in excess (Voet and Voet, 1995). Porphyrins follow a biliary route of excretion (Duffus and Worth, 1996), and may be quantified readily in fecal samples with high-performance liquid chromatography separation and fluorescence detection (Lim, 1991). Elevated levels of porphyrins, therefore, serve as useful biomarkers of the effects of contaminants on heme metabolism and have been used in numerous toxicological studies in both laboratory and field settings (DeMatteis and Lim, 1994).

In our field study to assess the effects of oil on heme metabolism in river otters in Prince William Sound following the *Exxon Valdez* oil spill, we observed higher levels of Copro III in fecal samples of river otters from oiled sites compared with nonoiled sites. We hypothesized that exposure to crude oil would result in higher levels of Copro III in fecal samples of river otters receiving doses of oil during the dosing period. We tested this hypothesis by comparing the patterns of porphyrin excretion in male river otters experimentally exposed to oil with those we observed in our field study.

6.2 METHODS

Experimental Conditions

River otters were live-captured in northwestern Prince William Sound, Alaska, USA, from late April to late May 1998, using No. 11 Sleepy Creek® leg-hold traps (Blundell et al., 1999) under permits from the Alaska Department of Fish and Game (98-001/003). The male river otters were held in captivity at the Alaska Sea Life Center (ASLC), Seward, Alaska, USA, from May 1998 to March 1999. Otters were fed frozen fish on a daily basis and their diet was supplemented with live fish. Initially, vitamins were provided with food, but because the river otters seemed reluctant to consume those fishes, B-complex vitamins (0.5 cc) were injected intramuscularly during the sampling sessions, which took place every three weeks. Minerals were provided to the river otters at all times in the form of a mineral block. The experimental river otters were allowed to acclimate to the conditions of captivity for approximately 90 days prior to oil dosing. During that time, average daily intake of food for the river otters was monitored to quantify the amount of oil required for achieving each level of oiling. At the end of the acclimation period, otters were randomly assigned to three experimental groups of five individuals each: 1) a control group that received no oil; 2) a low-dosage group that received 5 mg/kg body mass per day of oil; and 3) a high-dosage group that received 50 mg/kg body mass per day of oil. The oil used for this study was North Slope crude oil obtained from Williams, Inc.,

Fairbanks, Alaska, USA. Weathering of the oil was simulated in the laboratory through continuous mixing in sea water for 10 days at 25°C. Two batches of oil were weathered separately and the compositions of each batch were compared (Auke Bay Laboratory, NOAA, Juneau, Alaska, USA). Those batches differed slightly in composition, but both were comparable to the profile of oil spilled by the tanker vessel *Exxon Valdez* shortly after reaching the shorelines of the sound in 1989 (Short et al., 1996). The weathered oil was separated from the water and administered to the river otters in gel capsules hidden in fish every other day throughout the dosing period. Oil quantity was measured with a micro-pipette (Rainin Instruments Co., Emeryville, CA, USA) and weighed on a micro-balance. Occasionally the otters bit into the capsule and dropped the fish. When this occurred, oil was administered again during the following feeding to ensure that each otter ingested the amount of oil required for the experiment. Feeding of oil lasted 100 days from 21 August to 28 November 1998. Data collection continued for an additional 100 days of rehabilitation. The river otters were then fitted with radio transmitters and released at their sites of capture in Prince William Sound. All methods were approved by Independent Animal Care and Use Committees at the University of Alaska Fairbanks (97-14) and the Alaska Sea Life Center (98-002) and were in keeping with practices approved by the American Society of Mammalogists (Animal Care and Use Committee, 1998).

Sample Collection and Preparation

Fecal samples from each experimental river otter were collected during the week preceding a biomarker sampling session. Fecal samples were collected every three weeks from 29 June 1998 until 12 January 1999. An additional sampling session took place on 22-24 February 1999, in conjunction with the fitting of the radio transmitters. Fecal samples deposited by river otters identified through an observation window were collected within 30 min of deposition, placed in Whirlpak® bags, and frozen at -70° C until analysis. Fecal samples representing the field sites in Prince William Sound and southeast Alaska (Fig. 6.1) were collected from specific locations called latrines, where river otters socialize

and deposit feces and anal secretion (Ben-David et al., 1998). The fecal samples were placed in plastic bags and stored in a freezer at - 70°C. Prior to porphyrin extraction and analysis, the fecal samples were lyophilized for 24 h.

Reagents and Standards

ACS-grade hydrochloric acid, sodium phosphate, acetonitrile, and acetone were used for porphyrin extraction from the fecal samples. ACS-grade ammonium acetate, and HPLC-grade methanol were used for HPLC analysis. Milli Q-18 water was used for all solvent preparations.

The porphyrin standards used for determination and quantification of fecal porphyrins included a standard chromatographic marker kit containing the number I isomers of 8, 7, 6, 5, 4 carboxylate porphyrins and mesoporphyrin IX, as well as coproporphyrin III, deuteroporphyrin IX and protoporphyrin IX. All porphyrin standards were purchased from Porphyrin Products, Logan, Utah, USA.

Equipment

Separation of porphyrins was facilitated by an HPLC system consisting of two Waters 510 pumps (Waters Corp., Milford, MA, USA) a Rheodyne 7125 injector valve equipped with a 5-uL injection loop (Rheodyne Corp., Cotati, CA, USA) and a 3 by 0.46-cm Luna C-18 column equipped with a Security Guard® cartridge system (Phenomenex Corp., Torrance, CA,USA). Porphyrins were detected with a McPherson FL-748 fluorescence detector equipped with a 405 nm excitation cut-off filter, a 620 nm interference emission filter with a 10 nm bandwidth and a red sensitive photomultiplier tube (McPherson Corp., Chilmsford, MA, USA). Baseline 810 and Maxima 820 chromatographic software (Waters Corp.) were used for system control and peak integration. Sample concentration was facilitated with 500-mg trifunctional C-18 (tC-18)

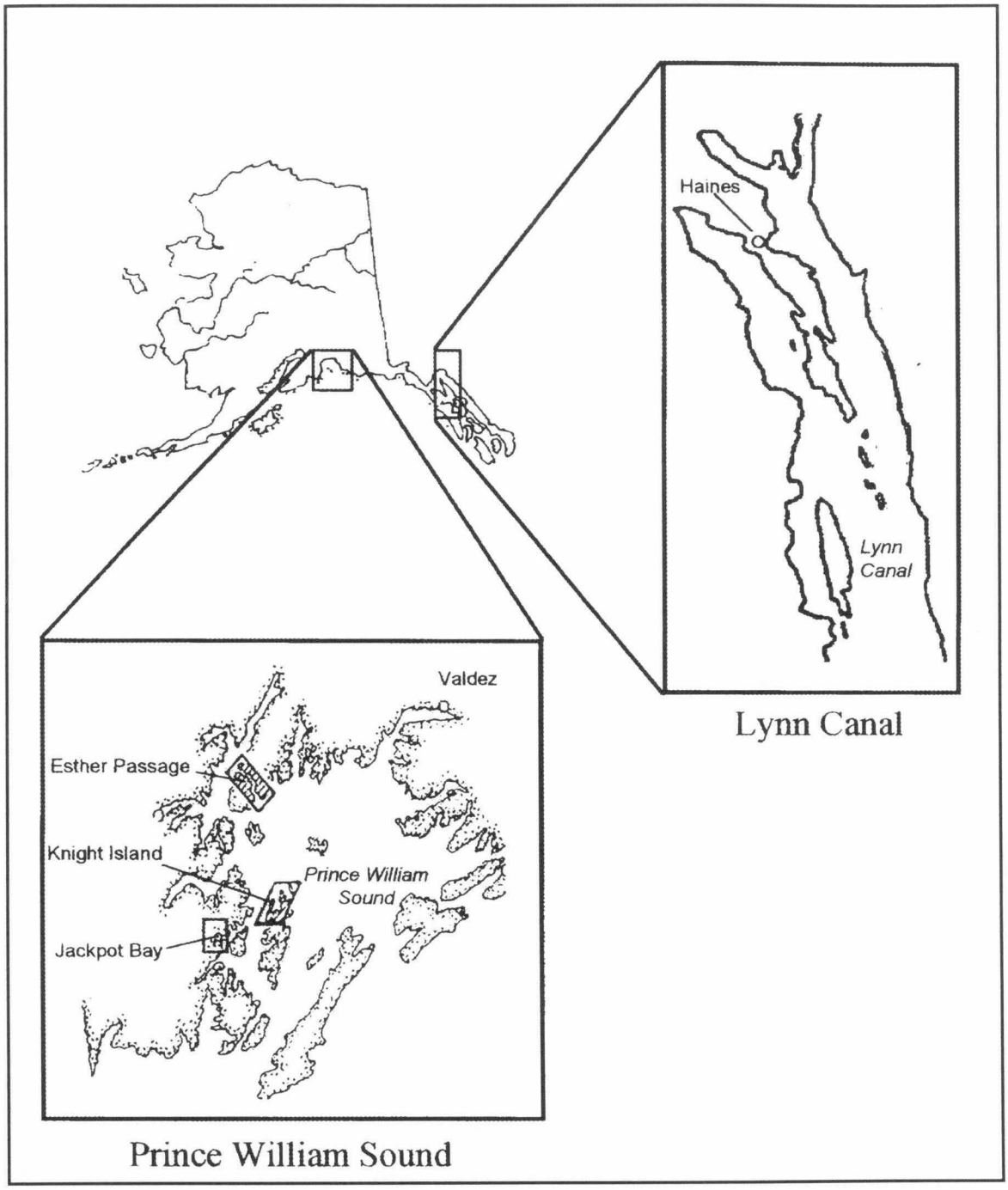

Figure 6.1. Field locations in Prince William Sound and southeast Alaska, USA, for river otter fecal porphyrin analysis.

solid-phase extraction columns (Sep-Paks®; Waters Corp., Milford, MA, USA) attached to a Supelco Visiprep vacuum manifold (Supelco Corp., Bellefonte, PA, USA).

Porphyrin Analysis

Porphyrin extraction and isolation from the fecal samples were accomplished with a modification of the procedure described by Bowers et al. (1992). Porphyrins were extracted with HCl, concentrated on the Sep-Pak® cartridges and eluted into cryogenic tubes with a polar solvent that was evaporated to produce a dry residue containing the porphyrins. Five milliliters of 6 N HCl were added to approximately 1.0 g of dry fecal material and the sample was macerated with a glass rod for approximately 1 min. The sample was mixed with a bench-top vortex for 1 min, sonicated in a water bath for 5 min, and mixed again for 1 min. Five milliliters of Milli Q-18 water were added to the sample. The sample was mixed again for 1 min and centrifuged for 10 min at 4,000 rpm. Two milliliters of the supernatant were delivered to a microcentrifuge tube and centrifuged at 7,000 rpm for 10 min.

Sep-Pak® cartridges were prepared by washing first with 7 ml of acetonitrile followed by 7 ml of sodium phosphate buffer (0.01 M, pH 3.5) after which 1.6 ml of the fecal supernatant were allowed to gravity feed. The Sep-Paks® were washed with 3 ml of sodium phosphate buffer (0.01 m, pH 3.5) to facilitate complete delivery of the supernatant to the Sep-Pak®, followed by 7 ml of sodium phosphate buffer (0.01M, pH 7.5) to enhance recovery of the porphyrins. The concentrate containing the porphyrins at the top of the Sep-Paks® was eluted into 5 ml cryogenic centrifuge tubes with 1 ml of acetonitrile, followed by 0.5 ml of acetonitrile : 1.0 N HCl (1:1, v/v), and finally 1 ml of acetone under vacuum. The tubes containing the eluates were placed in a water bath at 55°C and evaporated to a dry residue under a stream of air. The dry residues containing the porphyrins were stored frozen at -70°C. For HPLC analysis, the dry residues were reconstituted in 100 µL of 6 N HCl and injected directly into the HPLC system.

HPLC Analysis

The HPLC method used was a modification of the procedure described by Kennedy and James (1993). Separation of porphyrins was facilitated with a 6-min gradient elution and a two-component mobile phase consisting of ammonium acetate (1.0 M, pH 5.16) as solvent A, and 100% methanol as solvent B. Gradient elution commenced upon injection and proceeded from 25% B at time zero to 50% at 1.0 min, then to 95% B at 4.0 min, remained at 95% B for 1.5 min and returned to 25% B at 6 min. The column was allowed to re-equilibrate for 5 min at 25% B before the next injection. The concentration of porphyrins in each fecal sample was calculated with a seven-point calibration curve (0.0 μM to 3.0 μM) developed with solutions of porphyrin standards dissolved in 6 N HCl.

Statistical Analysis

A repeated measures multivariate analysis of variance model (MANOVAR) was used to evaluate differences in Copro III and Proto IX excretion between treatment groups of otters throughout the experimental period (PROC GLM; SAS, SAS Institute, Cary, NC, USA). The k nearest-neighbors randomization test (Rosing et al. 1998) was used to test for differences in Copro III and Proto IX excretion between the dosing periods and nondosing periods for all three treatment groups as well as between the control and high- dosage groups during the dosing period. Regression analysis (PROC GLM; SAS) was used to evaluate relationships between values of Copro III and Proto IX. The results obtained from one otter from the high-dosage group were excluded from statistical analysis because this otter was involved in a surgical procedure during the middle of the dosing period. The high-dosage group was therefore represented by 4, instead of 5 individuals. One tailed tests were conducted to test for differences in porphyrin excretion between treatment groups. For all statistical tests, the significance level was 0.05.

6.3 RESULTS

The porphyrin profiles in the fecal samples collected from the experimental river otters were characterized primarily by Uroporphyrin I (Uro I), Coproporphyrin I (Copro I), Coproporphyrin III (Copro III), and Protoporphyrin IX (Proto IX). Levels of Copro III and Proto IX were higher in fecal samples collected from the control group than in fecal samples collected from two different field sites (Table 6.1).

Table 6.1. Mean levels of porphyrins measured in fecal samples collected from river otters experimentally exposed to weathered crude oil. Levels of porphyrins measured during the first three sampling sessions of the experimental period are compared to the mean levels of porphyrins in fecal samples collected from two different field sites.

	Mean (nmol/g)				
Porphyrin	Control	Low Dose	High Dose	Jackpot Bay [a]	Lynn Canal [b]
Uro I	0.03	0.02	0.02	0.18	0.04
Copro III	1.31	0.95	1.13	0.54	0.50
Proto IX	4.71	4.95	4.48	2.31	1.90

[a] Field site in Prince William Sound Alaska, USA
[b] Field site in Northern Southeast Alaska, USA

Conversely, levels of Uro I were lower in fecal samples collected from the experimental river otters than in fecal samples collected at the field locations (Table 6.2).

Table 6.2. Detection of Uroporphyrin I in fecal samples collected during the dosing experiment and from two field locations.

Location	Percentage of Fecal Samples	Mean Level (nmol/g)
Dosing Experiment (n =161)	32	0.02
Jackpot Bay[a] 1996 (n = 23)	78	0.18
Lynn Canal[b] 1998 (n = 24)	38	0.04

[a] Field site in Prince William Sound Alaska, USA
[b] Field site in Northern Southeast Alaska, USA

No statistically significant differences in levels of Copro III or Proto IX between treatments groups were measured with the MANOVAR model ($F = 0.1767$, $p = 0.9896$,

df = 22, 2) and (F = 0.7198, p = 0.7297, df = 22, 2), respectively. Likewise, a k nearest-neighbors randomization test detected no differences in distribution of Copro III and Proto IX between the dosing and non-dosing periods or between the control and high dosage groups during the dosing period. No statistically significant differences were measured between the dosing and nondosing periods for the control, low-dosage, and high-dosage groups (p = 0.9993, 1.000, 0.4386, respectively) or between the control and high-dosage groups during the dosing period (p = 0.7145). Regression analysis revealed no relation between levels of Copro III and Proto IX for any of the treatment groups (p = 0.124, r-square = 0.0149).

6.4 DISCUSSION

Mean levels of Copro III in the fecal samples collected from the control river otters for the entire experimental period (1.31 nmol/g) were similar to mean levels of Copro III in fecal samples collected from an oiled field location in Prince William Sound one year following the *Exxon Valdez* oil spill (1.13 nmol/g). The higher mean levels of Copro III measured in the control river otters, therefore, precluded the establishment of a verifiable, baseline for comparison with the low-dosage and high-dosage treatments.

This research supports the concept of measuring the entire profile of porphyrins because of the additional information the profile provides. It is unlikely that the higher levels of Copro III and Proto IX measured in the fecal samples collected from the experimental river otters, but not observed in field studies, were caused by the experimental samples being of higher quality. The percentage of fecal samples containing detectable levels of Proto IX, which is rapidly degraded by bacteria (Beukeveld et al., 1987), was similar in samples from the experimental river otters (85%) and from the Lynn Canal reference site (79%). Nearly 100% of the samples from all three sampling locations contained detectable levels of Copro III and the levels of Copro III in samples collected from both the Lynn Canal and Jackpot Bay reference areas were similar. These results

indicate that the presence of Proto IX in the fecal samples may be a useful indicator of the age of the samples and that it is possible to collect samples of high quality for porphyrin analysis from the field. Although these results indicate that recovery of Copro III may be less dependent on the age of the sample than Proto IX, quantification of both Copro III and Proto IX in the same sample allows a more detailed evaluation of alteration of heme metabolism by focusing on coproporphyrinogen oxidase activity. These results support the establishment of a baseline for porphyrin excretion in marine populations of river otters represented by the porphyrin levels measured in fecal samples collected at both the Lynn Canal and Jackpot Bay reference areas.

Several factors may have contributed to the lack of observable differences in porphyrin levels between treatment groups. Elevated excretion of Copro III and Proto IX associated with captive conditions, such as a change in activity levels, diet or other captivity related physiological stress, may have masked more subtle changes in porphyrin excretion caused by effects of oil exposure. Changes in hepatic porphyrin excretion described by increases in the ratio of Copro III to Proto IX have been reported in fasting animals (Smith and El-Far, 1980). Also, variability inherent in individual porphyrin excretion as indicated in Fig. 6.2, as well as potential variability in individual response to oil exposure may have obscured the observation of any changes in porphyrin excretion that may have indicated oil-related effects.

Despite conducting a power analysis to minimize the impact of this research on Prince William Sound river otters, our results failed to demonstrate a statistically significant dose-response link between oil contamination and alteration of heme metabolism. Although examination of Fig. 6.2 shows a trend of lowering Proto IX levels in the high-dosage group compared to the low-dosage and control groups, without a statistically significant experimental verification of a causal link between oil exposure and

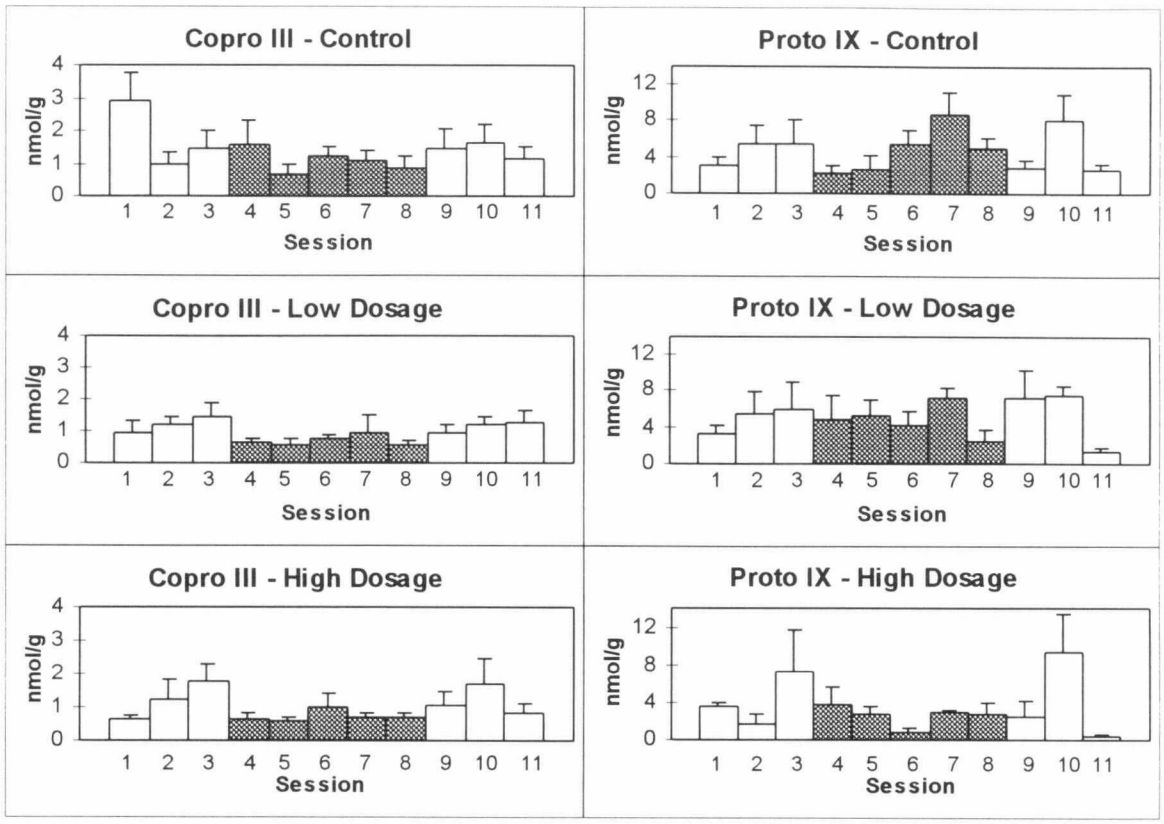

Figure 6.2. Mean levels (± SE) of Coproporphyrin III and Protoporphyrin IX measured in fecal samples collected from river otters experimentally exposed to North Slope crude oil. Sessions four through eight (grey bars) represent sampling sessions corresponding to the oil dosing phase of the experiment.

changes in porphyrin excretion, interpretation of field results based on this captive animal experiment should be approached conservatively.

This dosing experiment provided an unprecedented evaluation of the effects of oil exposure on heme metabolism in river otters under experimental conditions. These results suggest that fecal porphyrin excretion in river otters may be effected by physiological changes related to conditions of captivity and that further research is necessary to establish a reliable baseline for porphyrin excretion in river otters under experimental conditions to extrapolate from specific adverse health effects in individuals to effects observed at the population and ecosystem levels (Peakall, 1992). The noninvasive nature of fecal sample collection and availability of rapid analytical techniques warrants continued investigation of the use of the porphyrin profile in fecal samples of river otters as a biomarker of the effects of contaminant exposure in the marine coastal environment.

CONCLUSIONS

The results of this research project demonstrated that the porphyrin profile can be reproducibly characterized in fecal samples of river otters with HPLC separation and fluorescence detection. The results of the comparison of the HPLC and spectrofluorometric methods for the characterization of porphyrins in river otter fecal samples indicated that HPLC is more effective for separating and quantifying porphyrins in a complex fecal matrix while also being time efficient. The analytical method for characterizing porphyrin profiles developed for this project, however, could be made even more rapid and sensitive by employing a more advanced fluorescence detection system which would eliminate the need to concentrate porphyrins with solid phase extraction procedures.

River otters inhabiting oiled areas of Prince William Sound, Alaska were listed as recovered in the January 1999 Restoration Plan issued by the Exxon Valdez Oil Spill Trustee Council (EVOS 1999). Biomarker studies measuring blood haptoglobins and body mass (Duffy 1994b) provided evidence for recovery. The results of this thesis research project describing the profiles of fecal porphyrins in river otters inhabiting oiled and nonoiled areas during 1990 and 1996 also support the recovery designation. The profile of fecal porphyrin excretion in river otters inhabiting an oiled area in Prince William Sound demonstrated a similar temporal pattern of physiological stress as that of other biomarkers measured in river otters at the same oiled site.

Mean levels of porphyrins were different between two reference areas, Esther Passage and Jackpot Bay, and the oiled area during 1996. Those differences may be due to natural environmental factors including nutritional status or anthropogenic factors such as coastal land use, vessel traffic, and the presence of other contaminants. For example, timber harvests have taken place at the northern areas of Esther Passage (Bowyer et al. 1995). Also, a study to evaluate hydrocarbon signatures in oil residues on beaches of Prince William Sound revealed the presence of oil residues from a different origin than the

oil spilled from the *Exxon Valdez* (Kvenvolden et al. 1993). Most significantly, a LANDSAT Thematic Mapper image taken of Prince William Sound 2 weeks following the oil spill showed oil at the southern terminus of Esther Passage. Some of the animals sampled at Esther Passage may have been exposed to this oil. Therefore, differences in biomarker measurements observed between reference areas may be indicative of adaptation to pre-existing levels of environmental stress (Fossi and Leonzio 1994) as well as direct contact with *Exxon Valdez* oil.

To help establish a reliable baseline for porphyrin excretion in fecal samples of river otters that would facilitate the evaluation of oil related effects, profiles of porphyrins were characterized in fecal samples collected at coastal areas in northern Lynn Canal, southeast Alaska. The percentage of fecal samples collected in Lynn Canal containing detectable levels of Proto IX (79%) was similar to the percentage of fecal samples containing detectable levels of Proto IX (85%) collected immediately following deposition during the oil-dosing experiment at the Alaska Sea Life Center, Seward, Alaska. Conversely, the percentage of fecal samples collected during 1996 at the Jackpot Bay reference area in Prince William Sound containing detectable levels of Proto IX was only 28%. Nearly 100% of the samples from all three sampling locations contained detectable levels of Copro III. Furthermore, the levels of Copro III in samples collected from both the Lynn Canal and Jackpot Bay reference areas were similar. These results indicate that the presence of Proto IX in the fecal samples may be a useful indicator of the age of the samples and that it is possible to collect samples of high quality for porphyrin analysis from the field. Although these results indicate that recovery of Copro III may be less dependent on the age of the sample than Proto IX, quantification of both Copro III and Proto IX in the same sample allows a more detailed evaluation of alteration of heme metabolism by focusing on coproporphyrinogen oxidase activity. Finally, these results support the establishment of a baseline for porphyrin excretion in marine populations of river otters represented by the porphyrin levels measured in fecal samples collected at both the Lynn Canal and Jackpot Bay reference areas.

The oil-dosing experiment conducted at the Alaska Sea Life Center, Seward, Alaska designed to evaluate the response of river otters to diet related oil exposure did not facilitate the validation of the use of the porphyrin profile in fecal samples as a biomarker of the effects of oil exposure. Although no statistically significant differences in porphyrin excretion were detected between treatment groups, the lack of a reliable baseline for porphyrin excretion represented by the control group precluded the interpretation of porphyrin excretion in the fecal samples collected from the low dose and high dose groups during the dosing period. Mean levels of Copro III and Proto IX in the fecal samples collected from the river otters in the control group were higher than levels of Copro III and Proto IX measured in fecal samples collected from river otters in the low dose and high dose groups as well as fecal samples collected at the same time of year from the Lynn Canal and Jackpot Bay field reference sites.

The results of this research contribute to a gross evaluation of the physiological condition of river otters following the *Exxon Valdez* oil spill. Without the experimental verification of a causal link between oil exposure and changes in porphyrin excretion, however, interpretation of the field results should be approached conservatively.

One of the primary questions underlying the research objectives of the Near Shore Vertebrate Predator Program as stated in a recent project report (Holland-Bartels et al. 1998), "Is it oil or is it food?", remains persistent with regard to the results of this study. Increases in Copro III excretion in river otters inhabiting oiled areas may have been caused by poor nutrition as a result of an oil related decrease in prey availability or foraging ability. Changes in hepatic porphyrin excretion described by increases in the ratio of Copro III to Proto IX have been described in fasting animals (Smith and El-Far 1980). Indeed, changes in prey use as determined through analysis of fecal remains (Bowyer et al. 1994) as well as decreases in body mass (Duffy et al. 1993) of river otters in oiled areas were documented. Those changes may have been indicative of a lowered nutritive condition, which may have resulted in altered heme metabolism that lead to elevated excretion of Copro III rather than direct alteration of enzyme function by specific oil metabolites.

The establishment of normal physiological conditions in river otters in a captive environment is necessary before the cause and effect relationship between oil exposure and alterations of heme synthesis can be evaluated with measurements of fecal porphyrins. This should precede evaluation of the extrapolation from biomarker response, in this instance porphyrin excretion, to specific adverse health effects at the individual, population, and ecosystem levels (Peakall 1992). The results of this thesis research combined with the noninvasive nature of fecal sample collection and availability of rapid analytical techniques warrants continued investigation of the use of the porphyrin profile in fecal samples of river otters as a biomarker of the effects of contaminant exposure in the marine coastal environment.

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